# Isoenzymes in different tissues and cultured cells in genetically determined human diseases.

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ABSTRACT.

The component forms of three hydrolytic enzymes, N-acetyl-β-D-hexosaminidase, α-D-glucosidase and β-D-glucosidase, were studied in certain tissues, cultured cells and fluids. The possibility that hexosaminidases A and B had a common subunit and differed by a subunit, which was also a constituent of the neutral component, hexosaminidase C, was investigated and it was found that hexosaminidase C was unlikely to be related to either of the two major hexosaminidase components. The properties of another minor component, hexosaminidase S, suggested that this may contain the unique subunit of hexosaminidase A.

In addition to the α-glucosidase component deficient in Pompe's disease, three other apparently genetically unrelated components were identified. One of these, which had a neutral pH optimum and little activity at pH 4.0 occurred in most tissues, but was very labile. The others, one found in amniotic fluid and the other in kidney and leucocytes, although having neutral pH optima, had considerable activity at pH 4.0, thereby interfering with the diagnosis of Pompe's disease.

The enzyme found to be deficient in Gaucher's disease was associated with the β-glucosidase activity eluting in the void volume of Sephadex G-150. Two other more neutral components were detected, one of which was also in the void volume and the other, only present in some livers and in spleen, was of lower molecular weight. Neither of these components was deficient in either of the two cases of neuronopathic Gaucher's disease studied.

Methods were developed for detecting enzyme deficiencies

when there were other components with hydrolytic activity towards the artificial substrate, in the tissue being used. The DEAE-cellulose batch method was the most useful for detecting hexosaminidase A. Interfering α-glucosidase components were removed by isoelectric precipitation at pH 5.0 or the activity of the acid α-glucosidase was determined indirectly using equations derived on the basis of the relative susceptibility of components to inhibitors. Non-specific β-glucosidases residual in Gaucher tissues could be removed by preincubation in 50mM-sodium chloride buffered at pH 4.0.

DECLARATION.

I declare that, apart from the assistance acknowledged, I have carried out this work myself and this thesis is of my own composition.

D.M. Broadhead

ABBREVIATIONS.

These non-standard abbreviations were used in the thesis as follows:-

| | |
|---|---|
| PMN | Polymorphonuclear cells. |
| Hex- | Hexosaminidase component. |
| 4-MU | 4-methylumbelliferyl-. |

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# GENERAL INTRODUCTION

GENERAL INTRODUCTION.

Inherited diseases in which certain intermediates of metabolism are either stored in tissues or excreted in excess in the urine are usually caused by a deficiency of a particular enzyme as proposed by Garrod (1923). Subsequently, the concept that each gene was responsible for one enzyme was formally proposed (Beadle and Tatum, 1941). An important group of these diseases is that in which lysosomal enzymes are affected.

These enzymes, which have an acid pH optimum, are as their name suggests located primarily within the lysosome, thus protecting the cell from their destructive properties. Complex biological molecules are taken up by the lysosomes to be broken down to smaller molecules, and failure to degrade such complex molecules, owing to a deficiency of a particular enzyme activity, may lead to their storage in the lysosome (Hers, 1973). Diseases which have been attributed to deficiencies of particular lysosomal enzymes include the Lipidoses, Glycogenosis Type II and the Mucopolysaccharidoses.

The activities of many of the lysosomal enzymes may be assayed using artificial substrates (Leaback, 1974). These mimic the natural substrate and when acted upon by the enzyme, give rise to a chromagen or fluorogen, the concentration of which may be measured using a spectrophotometer or fluorimeter respectively, Artificial substrates, although easy to use, are not very specific and several enzyme components may attack one substrate, but have natural activities towards different substrates. With some diseases, it has so far only been possible to detect the

enzyme deficiency using the natural substrate. An example of this is Krabbe's Globoid Cell Leucodystrophy in which there is a deficiency of galactocerebroside β-galactosidase (Suzuki and Suzuki, 1970). There is no deficiency of non-specific β-galactosidase and the relationship between the specific and non-specific enzyme forms is uncertain (Suzuki and Suzuki, 1974a and b; Besley, 1975). The development of more complex artificial substrates may lead to the spectrophotometric assay of these specific enzymes (Gal et al., 1977).

The lack of specificity of a substrate allows the assay of several enzyme components. These have been referred to as isoenzymes. Although more than one enzyme component may attack an artificial substrate, not all of these may attack the same natural substrate. More than one enzyme component may be specific for the same natural substrate and these too may be described as isoenzymes. Given a definition based on function alone, isoenzymes may have a common genetic origin or be totally unrelated. The term, isoenzyme, is not very satisfactory and should be used with caution. It has been suggested (Leaback, 1976) that the term, isoenzyme, should be used only for enzymes attacking the same natural substrate and that the term, enzyme component, should be used when describing activities against artificial substrates.

Enzyme components may be tissue-specific. This may lead to difficulties in diagnosis using certain tissues if interference should occur from enzyme components, which have no activity towards the natural substrate. This problem was encountered with α-glucosidase in leucocytes (Koster et al., 1974) and will be

discussed in more detail later. Enzymes with similar natural function also differ from tissue to tissue. An example of this is the different forms of hexosaminidase A found in tissues and serum (Ikonne and Ellis, 1973). A study of the interrelationships of these isoenzymes may prove to be of importance in understanding the necessity for such specialisation.

The choice of enzymes studied in this thesis was to some extent dependent on the availability of cells and post-mortem tissues from patients, who had enzyme deficiencies. These were N-acetyl-β-D-hexosaminidase (Tay-Sachs and Sandhoff's diseases), acid α-glucosidase (Pompe's disease) and acid β-glucosidase (Gaucher's disease). Although cells from patients with other diseases were available, the three enzyme systems chosen presented problems of different types, which required varied techniques for their investigation.

Two general approaches may be used when attempting to assay the enzyme component, deficient in the disease, when other non-specific forms may be present. In the first approach, used in this thesis for α-glucosidase and β-glucosidase, the components, which interfere with the assay, are removed. In the other approach, the component to be assayed is selectively removed and its activity calculated by difference. This method has been used for estimating the heat and acid labile component A of hexosaminidase (O'Brien et al., 1970; Saifer and Rosenthal, 1973).

Hexosaminidases A and B appeared to be genetically related (Sandhoff et al., 1971) and it was also claimed that in some cases of Pompe's disease (Angelini and Engel, 1972) and Gaucher's disease

(Kanfer et al., 1975), activities other than those of the acid components were affected, implying that these other forms and the acid forms may be related. The genetic relationships of components will be investigated as will the possible natural function of non-specific hydrolases. Examples of the diagnosis of each of the diseases, both antenatally (if encountered) and postnatally will be given in cases, which arose in the course of the study.

# PART 1

## N-ACETYL-β-D-HEXOSAMINIDASE

## AND ITS

## DEFICIENCY IN $G_{M2}$ GANGLIOSIDOSIS

## THE $G_{M2}$ GANGLIOSIDOSES AND THEIR ASSOCIATED ENZYME DEFICIENCIES.

In 1881, Tay, a British ophthalmologist reported changes in the macula in the eyes of an infant. He subsequently reported these changes in other patients, including three in the same family (Tay, 1884; 1892). This change, which is now described as the cherry red spot, is in fact an artifact of degeneration of the retina around this spot. Sachs, an American neurologist, reported a form of familial idiocy, associated with early blindness (Sachs, 1887; 1896). This became known as Tay-Sachs disease. The first recognised cases of this disease were in Jewish families, but, as more cases were reported, the disease was also found in non-Jewish families.

Klenk (1939) showed that there was an accumulation of gangliosides in Tay-Sachs brain and concluded that the disease was due to a disorder of sphingolipid metabolism. Gangliosides are complex glycosphingolipids that contain one or more sialic acid residues and are found primarily in the central nervous system. Svennerholm and Raal (1961) reported the presence of excessive amounts of monosialogangliosides in Tay-Sachs brain. This was shown (Svennerholm, 1962) to be attributable to the storage of $G_{M2}$ ganglioside, which was formed after the removal of the terminal galactose moiety from the major normal monosialoganglioside ($G_{M1}$). Furthermore, it was also shown that the level of the asialo form of $G_{M2}$ ganglioside ($G_{A2}$) was raised in Tay-Sachs brain. The interrelationship of the various sphingolipids is shown in the appendix (page 255).

It was expected that the storage of these materials would

eventually be found to be due to a deficiency of a particular catabolic enzyme. Frohwein and Gatt (1967) extracted such an enzyme, N-acetyl-β-D-hexosaminidase, from calf brain and this was found to degrade $G_{M2}$ ganglioside, extracted from Tay-Sachs brain. It was likely that patients with Tay-Sachs disease would be found to be deficient in N-acetyl-β-D-hexosaminidase activity. This was not so, however, apart from one exceptional patient, who also had storage of kidney globoside (Sandhoff et al., 1968).

Human hexosaminidase was found to exist as two major components, A and B (Robinson and Stirling, 1968) and this finding gave impetus to investigations into Tay-Sachs disease. Okada and O'Brien (1969) and Hultberg (1969) reported the deficiency of hexosaminidase component A in Tay-Sachs disease. Furthermore, it was shown that components A and B were able to degrade $G_{A2}$ and globoside, whereas only component A was able to degrade $G_{M2}$ (Sandhoff, 1970).

The two major forms of $G_{M2}$ gangliosidosis are type B and type O. The former is more common and is usually known as Tay-Sachs disease; the latter is known as Sandhoff-Jatzkewitz disease or simply Sandhoff's disease. It is possible that there might be some variation within these groups, but the major difference between the two forms of the disease is that Sandhoff patients have an almost total deficiency of N-acetyl-β-D-hexosaminidase, whereas Tay-Sachs patients lack only component A. A large proportion of Tay-Sachs patients are of Jewish descent, but Sandhoff patients are panethnic. Two variant forms of Tay-Sachs

disease have been reported. Sandhoff (1969) reported one case with increased activity of hexosaminidases A and B in brain. This patient was diagnosed clinically as Tay-Sachs disease and shown to store $G_{M2}$ and $G_{A2}$ but not globoside (Sandhoff et al., 1971). In juvenile Tay-Sachs disease the activity of component A (Hex-A) was partially reduced (Young et al., 1970).

It was suggested (Sandhoff et al., 1971) that there was some genetic relationship between components A and B. If this were not so, it would be necessary to postulate that Sandhoff's disease was the result of two separate deficiencies. As Sandhoff's disease accounted for approximately one third of the cases of $G_{M2}$ gangliosidosis and was not linked to the Jewish population, this was not a very likely explanation.

To explain the dual enzyme deficiency in Sandhoff's disease, several theories may be put forward. The genes for components A and B may have been adjacent on the same chromosome and the deficiency in Sandhoff's may have been caused by a deletion of all or part of both genes. The gene for Hex-B has been shown to be located on the fifth chromosome, whereas genes on the fifth and fifteenth chromosomes were necessary for component A (Lalley et al., 1974; Gilbert et al., 1975). It was possible that a deletion across two genes on chromosome 5 could have led to the deficiency in Sandhoff's disease. As yet, no cases have been reported, which were deficient in Hex-B only and so theories in which Hex-A was dependent on the presence of Hex-B were favoured.

One possibility was that Hex-B was an intermediate in the synthesis of Hex-A. This satisfied the condition that Hex-A was

present only when Hex-B was also present. If Sandhoff patients did not have active component B, then they could not be expected to make component A. Tay-Sachs patients, on the other hand, have Hex-B, but would lack the capacity to convert it to component A. Another suggestion (Tateson and Bain, 1971) was that in Tay-Sachs disease, Hex-B was altered sufficiently to block its conversion to Hex-A, but not to affect its enzyme activity.

In order to understand the possible relationship between hexosaminidases A and B, a study of their properties was undertaken (Robinson and Stirling, 1968; Sandhoff, 1968; Poenaru and Dreyfus, 1973). Hex-B was heat stable, moved slowly towards the anode on electrophoresis at pH 6.5, was unbound to DEAE-cellulose at pH 6.0 and had an isoelectric point of pH 7-8. Hex-A, although having a similar molecular weight to component B, was relatively heat labile, moved more rapidly towards the anode on electrophoresis, was bound to DEAE-cellulose and had an isoelectric point of pH 5-5.5.

To explain the difference in charge between the two components, it was suggested that Hex-A differed from Hex-B in its sialic acid content. To test this hypothesis, Hex-A was purified and treated with neuraminidase (Robinson and Stirling, 1968; Murphy and Craig, 1972). The product was found to have an electrophoretic mobility similar to that of component B and the theory that Hex-A was synthesised from Hex-B by the addition of sialic acid residues, gained some support. Subsequently it was found that the conversion of Hex-A to Hex-B was unrelated to neuraminidase activity (Tallman et al., 1974). Using neuramin-

idase prepared from Clostridium perfringens, it was observed that more N-acetyl-neuraminic acid was released when Hex-A was incubated with enzyme for one week at lower temperatures rather than at 37°C. Only at 37°C, however, was much Hex-B formed. This showed that the conversion of Hex-A to Hex-B was unrelated to the release of N-acetylneuraminic acid. Furthermore, it was shown (Carmody and Rattazzi, 1973) that using a preparation of Vibrio cholerae extract, containing neuraminidase, similar to that used by earlier workers, conversion occurred even after heat inactivation of the neuraminidase. The conversion was found to be due to the presence of a preservative, merthiolate, in the commercial Vibrio cholerae extract and that p-hydroxymercuribenzoate and silver ions were also able to convert Hex-A to Hex-B (Beutler Villacorte et al., 1975).

In another theory, it was proposed that hexosaminidases A and B shared a common subunit. The conversion of Hex-A to Hex-B would then be explained by dissociation into subunits, accelerated by the presence of -SH reagents. It was proposed that in Sandhoff's disease, the subunit, common to both enzyme components, was absent, whereas in Tay-Sachs disease, the deficiency was of a subunit of Hex-A alone. This theory gave rise to two possibilities, a two locus or a three locus model. In the two locus model, it was proposed that Hex-B was composed of several subunits of the same type, whereas Hex-A was composed of two types of subunit, one of which was also present in Hex-B. In the three locus model, Hex-A was composed of two different types of subunit, one in common with Hex-B and one unique. Component B also had two types,

one of which was unique.

| | Hex-A | Hex-B |
|---|---|---|
| Two locus model | $(\alpha\beta)_n$ | $(\beta\beta)_n$ |
| Three locus model | $(\alpha\beta)_n$ | $(\beta\gamma)_n$ |

These alternatives were discussed by Srivastava and Beutler (1973; 1974). In support of the three locus model, it was reported (van Someren and van Henegouwen, 1973) that in Chinese hamster-human fibroblast hybrids, hexosaminidases A and B could be lost independently of each other. Lalley et al. (1974) refuted this, however, and found that no hybrid clones had Hex-A activity in the absence of Hex-B. This later data supports the two locus model.

The subunit theory predicted that Sandhoff patients would still have the unique subunit of Hex-A and in search of this subunit, the residual hexosaminidase activity in Sandhoff tissues was investigated. An enzyme component attracting interest was Hex-C, which was first reported by Hoogwinkel et al. (1972) in studies on human brain. Poenaru and Dreyfus (1973) showed that Hex-C was present in all human tissues and was more anodic than Hex-A on "Cellogel" electrophoresis. It was, indeed, a very good candidate for the unique subunit in Hex-A. Sandhoff patients did have Hex-C (Galjaard et al., 1974) and it had been claimed that Tay-Sachs tissues were deficient in this enzyme (Hoogwinkel et al., 1972). These two findings were necessary conditions if the subunit, unique to Hex-A, was also in Hex-C. When Tay-Sachs fibroblasts were hybridised with Sandhoff fibroblasts, hybrid cells were obtained, which now had Hex-A as well as Hex-B and

Hex-C (Galjaard et al., 1974). This was not evidence in itself for the subunit theory, as any theory based on the premise that the lesions in Tay-Sachs and Sandhoff's disease were complementary, would be supported by this result. In view of these findings, the significance of Hex-C in the $G_{M2}$ gangliosidoses was investigated and the results are reported in this thesis.

It would now appear that Hex-C is present in Tay-Sachs tissues (Penton et al., 1975) as well as in Sandhoff tissues. Consequently it would seem unlikely to contain the subunit unique to Hex-A. This subunit is probably contained in another form of hexosaminidase, recently found in Sandhoff tissues and termed Hex-S (Beutler, Kuhl et al., 1975; Ikonne et al., 1975). In view of the possible significance of this component, its properties were determined and will be considered in this thesis.

With the discovery of the deficiency in Tay-Sachs disease came the development of routine techniques for the detection of Hex-A. Two approaches have been used when differentiating between components A and B. One approach was dependent on the lability of Hex-A relative to Hex-B on heat treatment (Robinson and Stirling, 1968) or at acid pH (Saifer and Rosenthal, 1973). Alternatively, the difference in isoelectric point between the two components was used (Sandhoff, 1968) and Hex-A was detected by ion-exchange methods (Young et al., 1970) or by electrophoresis (Robinson and Stirling, 1968). Methods of heat or pH inactivation have been adapted for the analysis of large numbers of samples (O'Brien et al., 1970; Saifer and Perle, 1974). This

was particularly useful for heterozygote screening in areas where the Jewish population was large.

Control tissues and serum were found to lose hexosaminidase activity when heated or preincubated at acid pH prior to assay, whereas in Tay-Sachs disease little or no activity was lost, owing to the absence of Hex-A and consequent preponderance of Hex-B. It was reported (Tallman et al., 1974) that, on heating, Hex-A was converted to Hex-B with some loss of activity. If Hex-A were converted to Hex-B, the percentage A would be underestimated. Electrophoretic methods provide a good visual demonstration of the presence or absence of Hex-A, but are not easily adapted to give a quantitative result, essential for heterozygote detection. If a quantitative result were required, either the electrophoretogram could be sliced into sections for assay or a preparative column could be used. Neither of these procedures is as straight forward as inactivation techniques. Similarly, isoelectric focusing may be used, either in polyacrylamide gels or for a more quantitative result in a sucrose gradient. Using this technique, it was possible to clearly separate components A and B as well as other minor components (Sandhoff et al., 1971). This again was not a very good routine method. The same might be said of ion-exchange methods. Dance et al. (1969) reported that Hex-B was was unbound to DEAE-cellulose, whereas Hex-A was bound but could be eluted with a linear gradient of sodium chloride. The method has been automated, however, to give it some of the advantages of the heat method but with a more accurate determination of Hex-A (Ellis, Ikonne and Masson, 1975). The automated

DEAE-cellulose column also provided information about other hexosaminidase components, undetected by inactivation methods. DEAE-cellulose could also be used in a batch method, which made use of the binding property of the resin in the test tube (Dance et al., 1970). Hex-B was not bound at low chloride concentration, whereas Hex-A was only free in the presence of higher concentrations of anion. This method proved to be particularly useful when small numbers of samples were being screened for Tay-Sachs disease. The different methods of determining Hex-A were compared in this study.

Minor components of hexosaminidase have been investigated. Price and Dance (1972) reported that two minor components were eluted between hexosaminidases A and B on DEAE-cellulose. These components, designated $I_1$ and $I_2$, were found particularly in serum instead of Hex-B. Stirling (1972) found a major intermediate component of hexosaminidase in the serum of pregnant women. This, he designated Hex-P and on the basis of its electrophoretic mobility, which was towards the anode but slower than Hex-A, its behaviour on DEAE-cellulose and heat stability, suggested that it might be identical with Hex-$I_2$.

Human serum Hex-A was shown to consist of two components (Ikonne and Ellis, 1973). The minor component was apparently identical to tissue Hex-A. The major Hex-A component of serum was eluted from DEAE-cellulose at a lower salt concentration than the tissue form and if multiple pass Sephadex G-150 gel filtration was employed, the major serum component was eluted before tissue

Hex-A. These workers also found that, although tissue Hex-A was unaltered after treatment with neuraminidase derived from Clostridium perfringens, serum Hex-A was converted to a form which was eluted from DEAE-cellulose earlier than the native enzyme. Serum Hex-A was not converted to tissue Hex-A by neuraminidase treatment and was not, therefore, merely a sialated form of tissue Hex-A. Hex-A was found to be deficient in the serum of patients with Tay-Sachs disease (Okada and O'Brien, 1969) and so serum and tissue forms appear to be closely related. The intermediates, now known as hexosaminidases $I_1$ and $I_2$, were present in the tissues of patients with Tay-Sachs disease (Young et al., 1970), so these were more likely to be related to Hex-B.

Ellis and Patrick (1976) have studied the distribution of hexosaminidases in human leucocytes. They found that the Hex-A peak of leucocytes on DEAE-cellulose was eluted similarly to that of grey matter, but that the peak was asymmetric, suggesting that another component, eluting just before this peak, was present. Examination of separated lymphocytes and polymorphonuclear cells (PMN), revealed that the Hex-A in PMN was eluted similarly to that total leucocytes, although the activity of Hex-B was much higher in the latter. The Hex-A in lymphocytes, however, was eluted after that of total leucocytes or PMN. The activity of Hex-B was higher in lymphocytes than in PMN and it was pointed out that problems could be encountered in screening for Tay-Sachs carriers using leucocytes, since the percentage Hex-A was dependent on the relative proportions of lymphocytes and PMN. The results of

isoelectric focusing studies, which led to a better resolution of the hexosaminidases of lymphocytes and PMN, are reported in this thesis.

METHODS.

Enzyme assay.

4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (0.5mM) (Koch-Light Labs. Ltd.) were the substrates for N-acetyl-β-D-glucosaminidase and N-acetyl-β-D-galactosaminidase respectively. For the standard assay, substrate at double the final concentration required, was dissolved in phosphate-citrate buffer (0.2M/0.1M), pH 4.5, (McIlvaine, 1921) containing Triton X-100 (0.1%$^{v}$/v) and sodium azide (0.02%$^{w}$/v). Buffer-substrate mixture (1 vol.) was mixed with extract (1 vol.) in an LP3 tube (Luckham Ltd.) and incubated at 37°C. After incubating the mixture for a known period of time, the reaction was stopped with 0.1M-sodium carbonate and the fluorescence read as described in the appendix (page 242).

For the pH profile, substrate dissolved in distilled water at four times the final concentration (1 vol.) was added to double strength buffer (1 vol.) and the assay was started with extract (2 vols.). Protein was determined (Lowry et al., 1951) as described in the appendix (page 243), using bovine serum albumin (Sigma Ltd.) as standard.

Enzyme source and preparation of extract.

(i) Cultured cells.

Amniotic fluid cells and skin fibroblasts were cultivated as described in the appendix (page 244). When it was important to remove the hexosaminidase activity of the fetal bovine serum in the medium, the serum was incubated at 60°C for two hours. The serum was filtered through a millipore filter before adding to the medium.

(ii) Blood samples.

Leucocytes were usually isolated using dextran as described in the appendix (page 247). This method preferentially isolated polymorphonuclear cells. If lymphocytes were required, these were isolated using Ficoll-Paque, also described in the appendix (page 248).

Serum was prepared by low speed centrifugation (900g) of clotted blood.

(iii) Post-mortem tissues.

Tissues, liver and brain, were stored at -40°C until required. These were obtained within 24 hours of death.

Cultured cells and white blood cells were disrupted by sonication using a "Soniprobe" (Dawe's Instruments) and centrifuged (900g for 5 mins.). Tissues were homogenised using either a Potter-Elvehjem all glass homogeniser or a mixer emulsifier (Silverson Machines Ltd.), sometimes followed by sonication to further disrupt the tissue.

Separation techniques.

(i) DEAE-cellulose.

DEAE-cellulose was prepared as described in the appendix (page 250) and equilibrated with 10mM-sodium phosphate buffer, pH 6.8, for all routine work. The sample was applied to a column (12 x 0.8cm. diam.) packed with DEAE-cellulose and unbound material eluted with 10ml buffer. Fractions (1ml) were collected. A linear chloride gradient was applied at a flow rate of 20ml/h and 40 fractions (1ml) were collected up to a chloride concentration of approximately 350mM.

For the DEAE-cellulose batch method, the following reagents were added to an LP3 tube (Luckham Ltd.).

| | Hexosaminidase | |
|---|---|---|
| | B | AB |
| DEAE-cellulose slurry | 0.3ml | 0.3ml |
| Cell extract | 0.05ml | 0.05ml |
| NaCl in buffer (0.32M) | 0.05ml | — |
| NaCl in buffer ( 2M ) | — | 0.05ml |

The tubes were capped and mixed ($4^{\circ}C$) on an angled rotary mixer for 15 minutes. After mixing, the slurry was separated from the supernatant by centrifugation (900g for 2 mins.). The supernatant was assayed for hexosaminidase activity and the percentage hexosaminidase A was calculated by difference.

(ii) Isoelectric focusing.

The method used for isoelectric focusing, an adaptation of that of Godson (1970), is described in detail in the appendix (page 251).

(iii)Electrophoresis.

Electrophoresis on "Cellogel" strips (Reeve Angel Scientific Ltd.) was by the method of Poenaru and Dreyfus (1973). The strips were wet with 40mM-potassium phosphate buffer, pH 6.5. Excess buffer was removed from the surface and sample (25µl) was applied using a Lang-Levy constriction pipette. A voltage of 200 volts (15 v/cm) was applied for two hours at 4°C.

At the end of the run, the strips were placed face down on Whatman 3MM chromatography paper, soaked in buffered substrate and incubated in a warm humid atmosphere. When it was adjudged that sufficient 4-methylumbelliferone had been released, the strips were exposed to ammonia vapour and the fluorescence visualised using a UV. light source.

Heat inactivation.

For the heat inactivation method for the estimation of percentage hexosaminidase A, extract was mixed with bovine serum albumin (BSA) and buffer as follows:-

| | enzyme | blank |
|---|---|---|
| Tissue or cell extract | 0.3ml | — |
| Distilled water | — | 0.3ml |
| BSA (3mg/ml) | 0.3ml | 0.3ml |
| Sodium citrate buffer (0.1M), pH 5.0 at 50°C | 0.6ml | 0.6ml |

These mixtures were incubated at 50°C and aliquots (100µl) were removed and stored at 4°C at intervals of 0, 10, 20, 30, 45, 60, 120 and 240 minutes. An aliquot (25µl) for each time point was assayed in duplicate for hexosaminidase activity. After

subtracting the blank activity, the $\log_{10}$ of the percentage activity remaining was plotted against time. The slope of the second phase of the biphasic plot was extrapolated to zero time to estimate the percentage hexosaminidase B in the original mixture and from this the percentage hexosaminidase A was calculated.

Initially ordinary bovine serum albumin (Sigma Ltd.) was used but this had too high a hexosaminidase activity for later experiments in which lower activities were being investigated. It was suggested (R.B. Ellis, personal communication) that a lower blank would be encountered if BSA fraction V was used but this too had an unacceptably high blank. It was, however, possible to separate the hexosaminidase activity in BSA fraction V from most of the protein by Sephadex G-150 gel filtration (Figure 1a, page 23). The column (35 x 3cm diam.) was eluted with 5mM-sodium phosphate buffer, pH 7.0, containing 5mM-NaCl, the hexosaminidase activity eluting after the major protein peak. The hexosaminidase-free BSA was dialysed against distilled water, concentrated by vacuum dialysis and lyophilised.

Not only had the purified BSA a low blank but gave better biphasic heat inactivation plots (Figure 1b, page 23). This was not attributable to the removal of bovine hexosaminidases, as the substitution of distilled water for BSA did not give a good biphasic plot either.

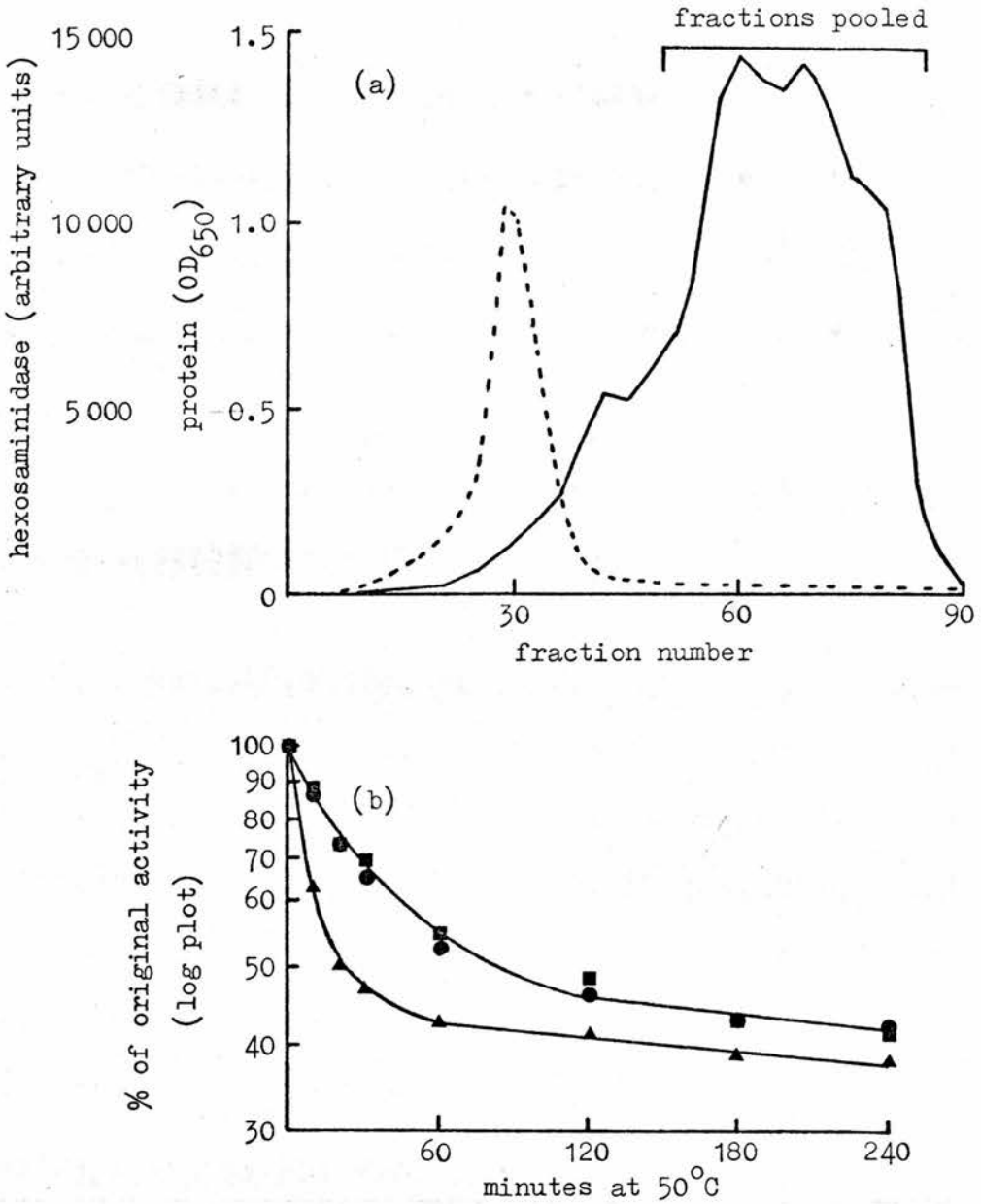


FIGURE 1. (a) Sephadex G-200 gel filtration of 500mg BSA fraction V to remove hexosaminidase activity.

——— protein ----- hexosaminidase

(b) Hexosaminidase heat inactivation plot for control liver using distilled water instead of BSA (●), BSA fraction V (■) and BSA fraction V after gel filtration (▲).

Preparation of hexosaminidase C.

Brain (80g) from a stillborn anoxic infant was homogenised using a mixer emulsifier (Silverson Machines Ltd.) in 10mM-sodium phosphate buffer, pH 6.0, containing Triton X-100 (0.1%$^{V}$/v) and sodium azide (0.02%$^{W}$/v). The homogenate was centrifuged (900g for 30 minutes at 4°C) and the supernatant was concentrated tenfold by vacuum dialysis. After centrifuging once more (10 000g for 30 minutes at 4°C), the supernatant (2ml) was applied to a Sephadex G-200 column (90 x 4.4cm diam.), equilibrated with the same buffer in which the tissue had been homogenised. The column was eluted at an ascending flow rate of 24ml/h and fractions (8.1ml) were collected. Fractions were assayed for N-acetyl-β-D-glucosaminidase activity at pH 4.5 and pH 5.5. The first peak had more activity at pH 5.5 than at pH 4.5, whereas the second peak had the greater activity at pH 4.5. Hexosaminidase C (peak I) and peak II were taken for further studies.

Preparation of hexosaminidase S.

Normal human liver (1g) was homogenised in 0.1%$^{V}$/v Triton X-100 (3ml) using a Potter-Elvehjem all glass hand homogeniser. The homogenate was then sonicated and centrifuged at 800g for 5 minutes (4°C). The supernatant was adjusted to pH 5.0 with sodium acetate buffer and centrifuged once more at 800g for 5 minutes (4°C). The supernatant (2.5ml) was dialysed at 4°C against 10mM-sodium phosphate buffer, pH 6.0, containing 0.05%$^{V}$/v Triton X-100 and 0.02%$^{W}$/v sodium azide. After low speed centrifugation, the supernatant was applied to a DEAE-

cellulose column (40 x 2cm diam.), equilibrated with the buffer used for dialysis. After twenty fractions (5ml) had been collected, a chloride gradient was applied to the column and a further eighty fractions (5ml) were collected. Selected fractions were taken from the tail of the hexosaminidase A peak, dialysed against distilled water and focused. Hexosaminidase S, relatively free of other hexosaminidase components was obtained.

(a) STUDIES ON HUMAN HEXOSAMINIDASES.

RESULTS.

(i) Binding of hexosaminidases to DEAE-cellulose.

When homogenates were applied to a DEAE-cellulose column, two major peaks of activity were eluted, corresponding to hexosaminidases A and B. A typical plot is shown in Figure 2 , where a homogenate prepared from cells cultured from human urine was used. The first major peak of activity, Hex-B, was eluted

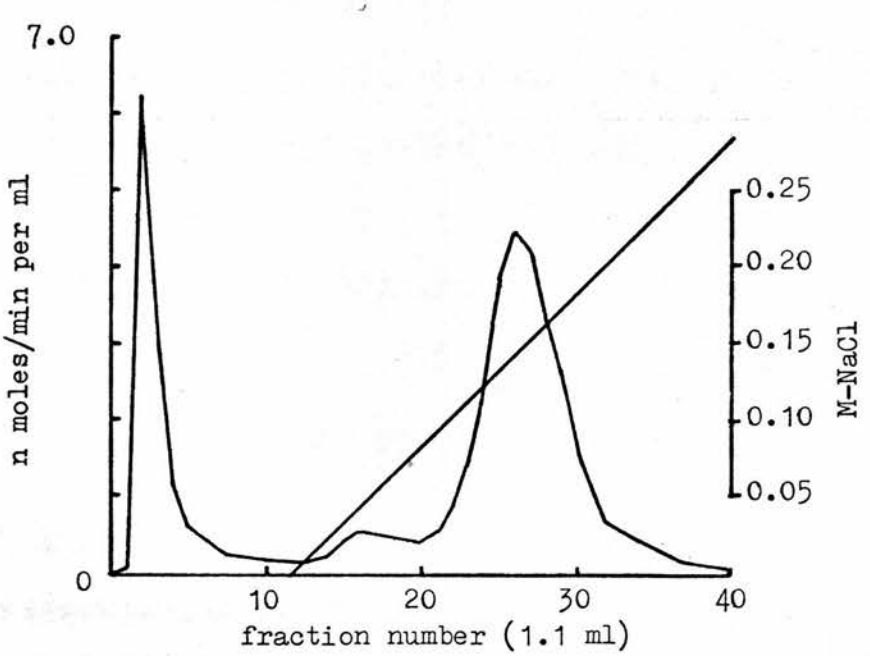


FIGURE 2. Separation of cultured urine cell hexosaminidases on DEAE-cellulose.

before the chloride gradient was applied and the larger peak, Hex-A, was eluted at 145mM-sodium chloride. A minor, broad peak of activity was eluted as soon as chloride was applied to the column. The percentage Hex-A was estimated by DEAE-cellulose column chromatography, the DEAE-cellulose batch method or by heat inactivation (Table 1 ). Using commercial lyophilised bovine serum albumin, the heat inactivation method consistently underestimated Hex-A activity, compared with the other two methods which gave closely similar results.

When the activity of hexosaminidase was measured in cultured cells, it was found that the activity of cultured skin fibroblasts was not greater than that of cultured amniotic fluid and urine cells (Table 2 , page 28 ). It was also found that the

TABLE 1 Comparison of the three methods used for the estimation of percentage hexosaminidase A.

| Cell extract | % Hex-A* | | |
|---|---|---|---|
| | Heat inactivation | DEAE-cellulose batch | DEAE-cellulose column |
| 1 | 58 | 77 | 74 |
| 2 | 53 | 80 | 80 |
| 3 | 60 | 71 | 72 |
| 4 | 53 | 69 | 65 |
| 5 | 46 | 73 | 73 |
| 6 | 41 | 48 | 48 |

* Activity of hexosaminidase A expressed as a percentage of total activity.

TABLE 2 Hexosaminidase activity in different cell types.

| Cell type (no. of samples) | Hexosaminidase activity n moles/min per mg of protein mean ±SD (range) | | | %A (range) |
|---|---|---|---|---|
| | Total | Hex-B | Hex-A | |
| Amniotic Fluid Cells (20) | 54.94 ±22.6 (18.13- 99.00) | 20.06 ±12.6 ( 6.93- 53.55) | 34.88 ±14.2 (11.20- 53.40) | 64 (42-82) |
| Urine Cells (14) | 53.52 ±19.2 (10.05- 89.65) | 19.36 ± 9.0 ( 5.68- 39.30) | 34.16 ±12.5 ( 4.37- 54.88) | 64 (44-75) |
| Skin Fibroblasts (20) | 64.25 ±35.9 (31.62-173.22) | 33.09 ±19.0 (17.49- 85.05) | 31.16 ±17.7 (13.54- 88.13) | 49 (39-62) |

percentage Hex-A, determined using the DEAE-cellulose batch method, was lower in cultured skin fibroblasts than in the other two cell types. As Hex-A had a similar specific activity in all three cell types, the greater total activity in cultured skin fibroblasts was attributable to Hex-B. A particularly interesting observation was that morphologically fibroblastic amniotic fluid cells did not differ from epithelioid cells with respect to the two major enzyme components.

(ii) Separation of hexosaminidases by isoelectric focusing.

Isoelectric focusing was the most effective means of separating the various component forms of hexosaminidase. The isoelectric points of hexosaminidases A and B of cultured skin fibroblasts were pH 5.5 and pH 8.2 respectively (Figure 3 ). No other components were seen. The isoelectric point of serum Hex-A was pH 5.0; several peaks with higher isoelectric points were also present (Figure 4a, page 31). When serum from a pregnant woman was focused, the major hexosaminidase component (Hex-P) had an isoelectric point of pH 6.5 (Figure 4b, page 31).

Isoelectric focusing of liver homogenates gave two major peaks of activity corresponding to the hexosaminidases A and B found in cultured skin fibroblasts (Figure 5 , page 32). Sometimes a smaller peak at pH 6.5 was also detected. The profile in brain was somewhat different. A neonatal brain

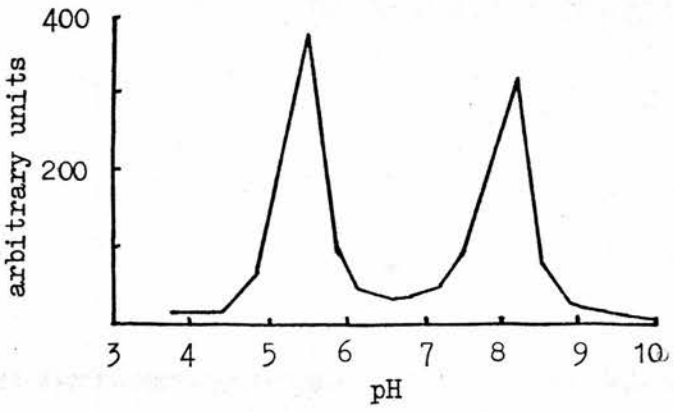


FIGURE 3 . Isoelectric focusing in the range pH 3.5-10.0 for control cultured skin fibroblasts.

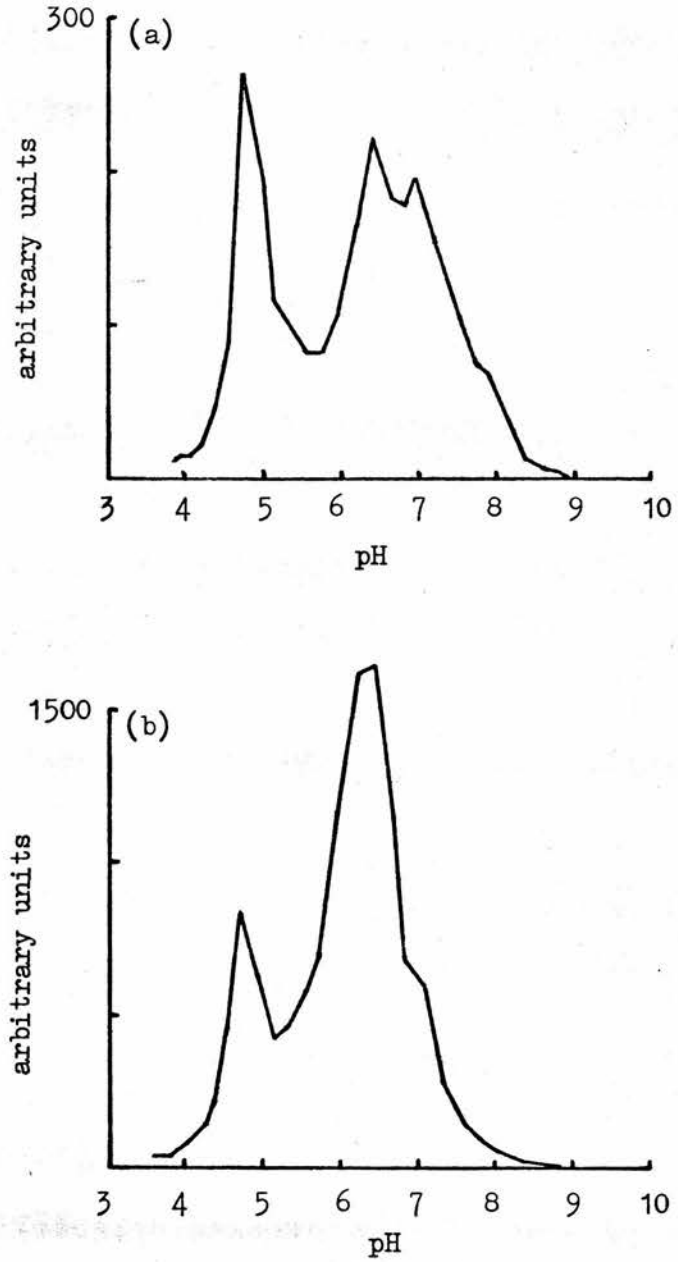


FIGURE 4 . Isoelectric focusing in the range pH 3.5-10.0 for (a) control human serum and (b) control human pregnancy serum.

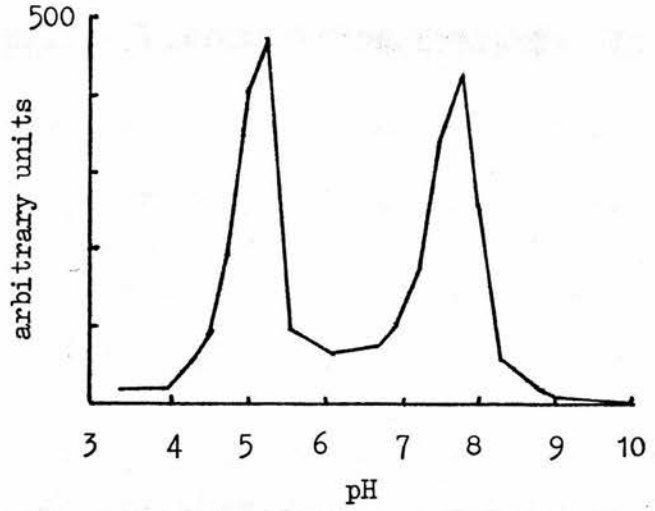


FIGURE 5. Isoelectric focusing in the range pH 3.5-10.0 for control human liver.

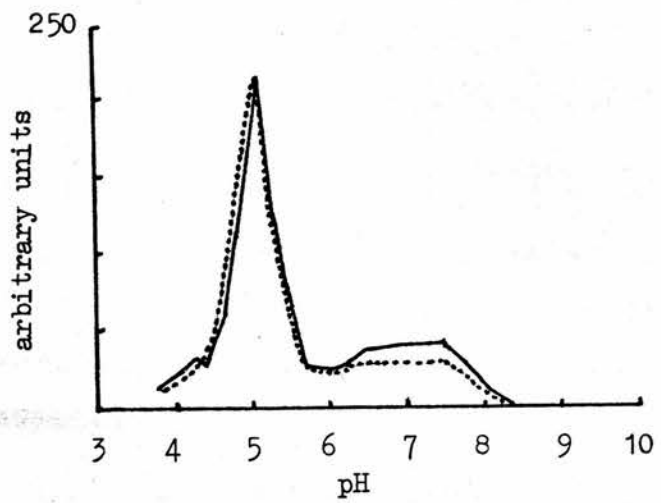


FIGURE 6. Isoelectric focusing in the range pH 3.5-10.0 for control neonatal human brain.

——— assayed at pH 4.5

- - - - - assayed at pH 5.5

homogenate was focused and gave rise to a sharp peak of hexosaminidase with pI 5.2, together with a broad band of activity which peaked at pH 6.5-7.5 (Figure 6, page 32). There was no peak corresponding to the hexosaminidase B normally present in cultured skin fibroblasts and liver. When assayed at pH 4.5, the peak with pI 5.2 was skewed towards a higher isoelectric point, but when the fractions were assayed at pH 5.5, this peak was skewed in the opposite direction.

Differences have been found in the hexosaminidase components of lymphocytes and polymorphonuclear cells (PMN) using DEAE-cellulose chromatography (Ellis and Patrick, 1976). Isoelectric focusing was thought to be a more useful tool for investigating these differences. The patterns for lymphocytes and PMN were not dissimilar (Figure 7, page 34). The major difference was that the peak with the lowest isoelectric point occurred in the PMN only. This had an isoelectric point around that of Hex-S (see Figure 16, page 44). Peak II, the major peak in the two lymphocyte preparations tested and in one of the PMN preparations (second largest to peak I in the other), corresponded very closely to serum Hex-A. Component IV was probably tissue Hex-A, whilst components III and V, which only appeared as shoulders, did not correspond to any previously designated hexosaminidase.

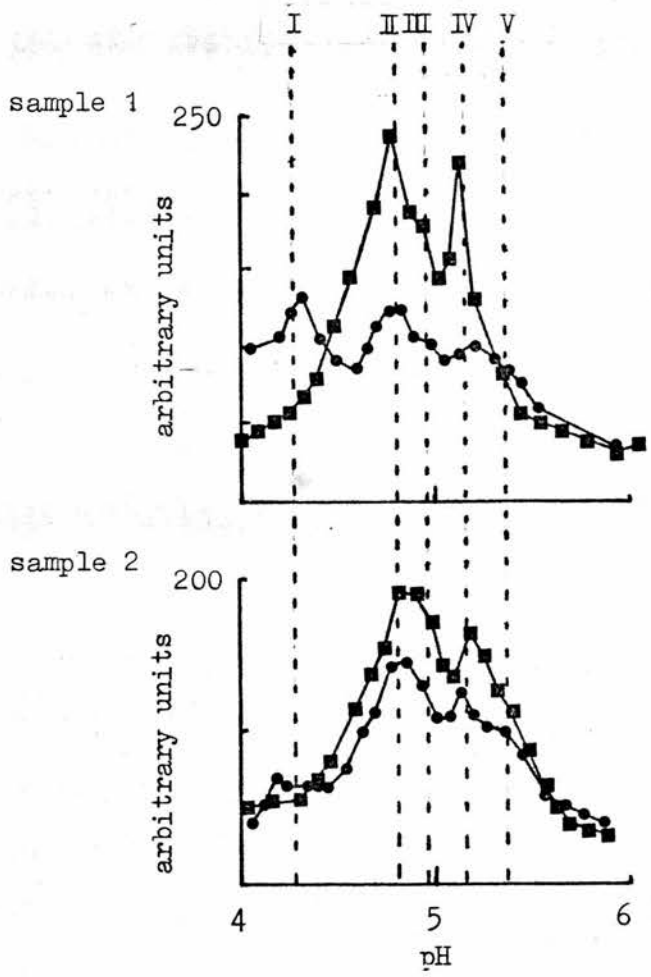


FIGURE 7 . Isoelectric focusing in the range pH 4.0-6.0 of lymphocyte (■) and polymorphonuclear cell (•) hexosaminidases from two control blood samples.

(iii)Hexosaminidase C.

Hexosaminidase C was obtained from the brain of a stillborn anoxic infant. The enzyme was separated from other hexosaminidase components by Sephadex G-200 gel filtration (Figure 8 ). The column had previously been calibrated using Blue Dextran and Hex-C was adjudged to be just included.

The isoelectric focusing profile of the first peak of hexosaminidase from the Sephadex G-200 column had a single peak

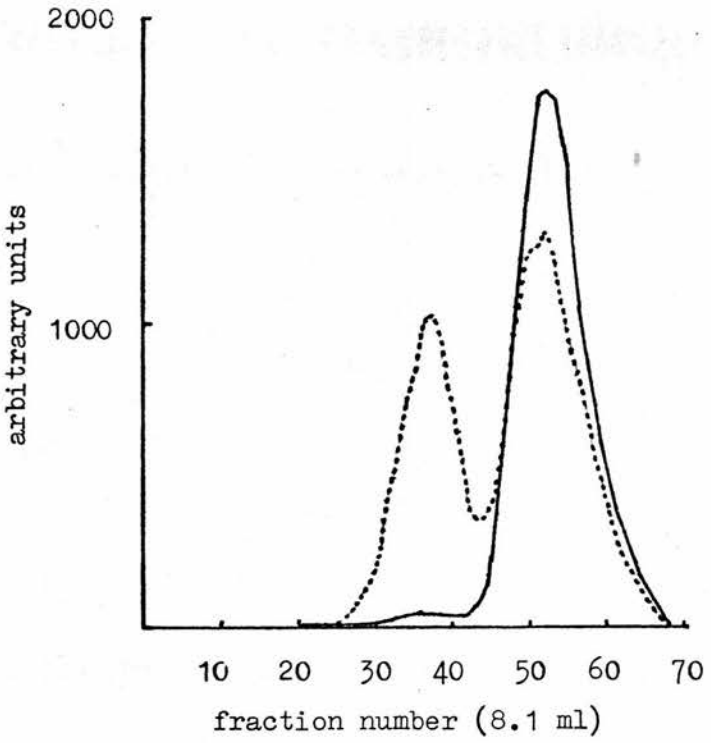


FIGURE 8 . Separation of neonatal human brain hexosaminidases by Sephadex G-200 gel filtration. 300 ml were collected before fraction 1.

———— assayed at pH 4.5

- - - - - assayed at pH 5.5

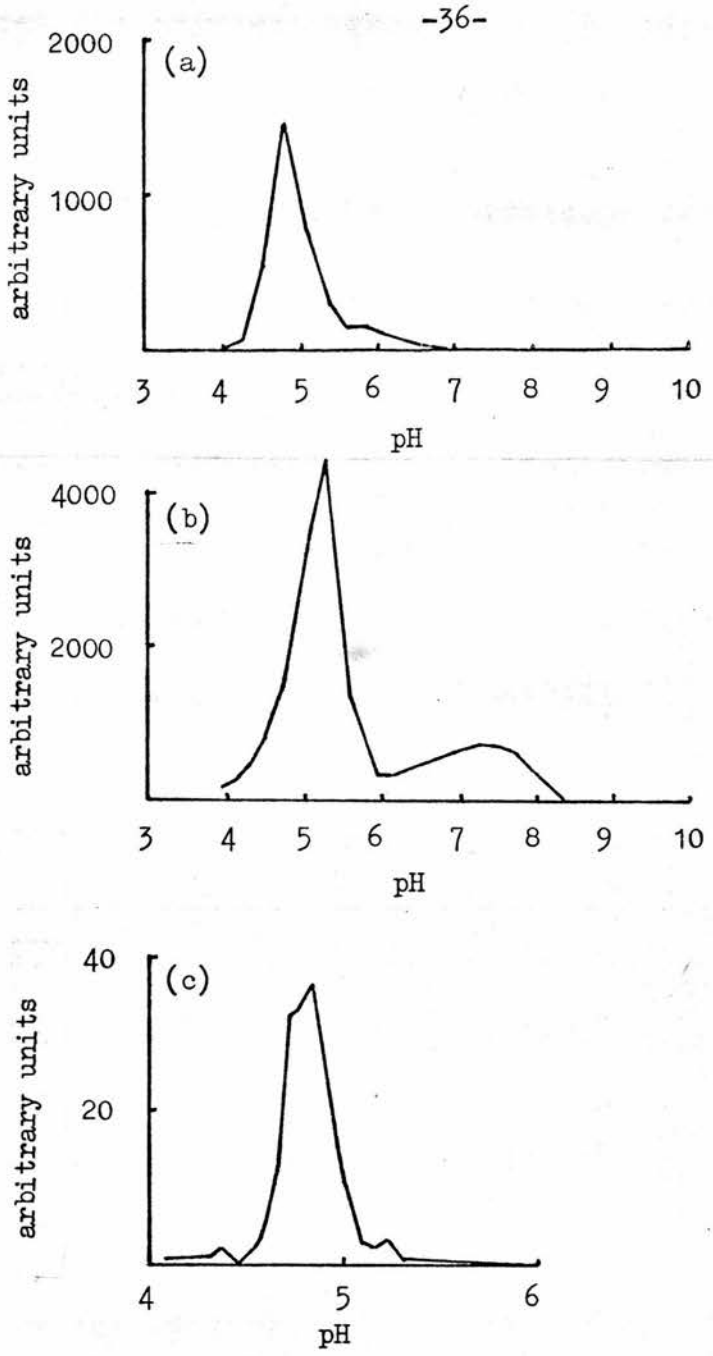


FIGURE 9 . Isoelectric focusing of the peaks of hexosaminidase obtained from Sephadex G-200 gel filtration of neonatal brain.

a) Peak I in the range pH 3.5-10.0 assayed at pH 5.5.

b) Peak II in the range pH 3.5-10.0 assayed at pH 4.5.

c) Peak I in the range pH 4.0-6.0 assayed at pH 5.5.

at pH 4.77 (Figure 9a, page 36). Peak II gave two focusing peaks with isoelectric points of pH 5.27 and pH 7.40, corresponding to tissue Hex-A and a component with an isoelectric point lower than that of the Hex-B of liver and cultured skin fibroblasts (Figure 9b, page 36). When peak I was focused in the range pH 4.0-6.0, the isoelectric point of the hexosaminidase peak was pH 4.80 (Figure 9c, page 36).

In order to show that peak I was the same as Hex-C described previously (Poenaru and Dreyfus, 1973), electrophoresis was carried out. Difficulties were encountered when concentrated peak I was applied to "Cellogel" strips, as the presence of residual Triton X-100 caused the sample to run on the surface of the strip. Prolonged dialysis against distilled water led to precipitation of the enzyme. Dialysis against 10mM-sodium phosphate buffer, pH 7.0, containing 0.1M-sodium chloride resulted in some loss of activity but after low speed centrifugation, the sample was suitable for electrophoresis. Peak I hexosaminidase was more anodic than Hex-A, corresponding to Hex-C (Figure 10 , page 38).

The pH profile of Hex-C had an optimum of pH 5.5-6.0 (Figure 11, page 39 ). The activity fell away sharply on the acid side of the peak and at pH 4.5 was only 11% of the maximum.

The apparent $K_m$ was determined for peaks I and II using both the glucosaminide and galactosaminide conjugates of methylumbelliferone. Assayed at pH 4.5, the apparent $K_m$s for peak II were 0.88mM and 0.116mM for glucosaminide and galactosaminide respectively, whereas the respective values for peak I (Hex-C)

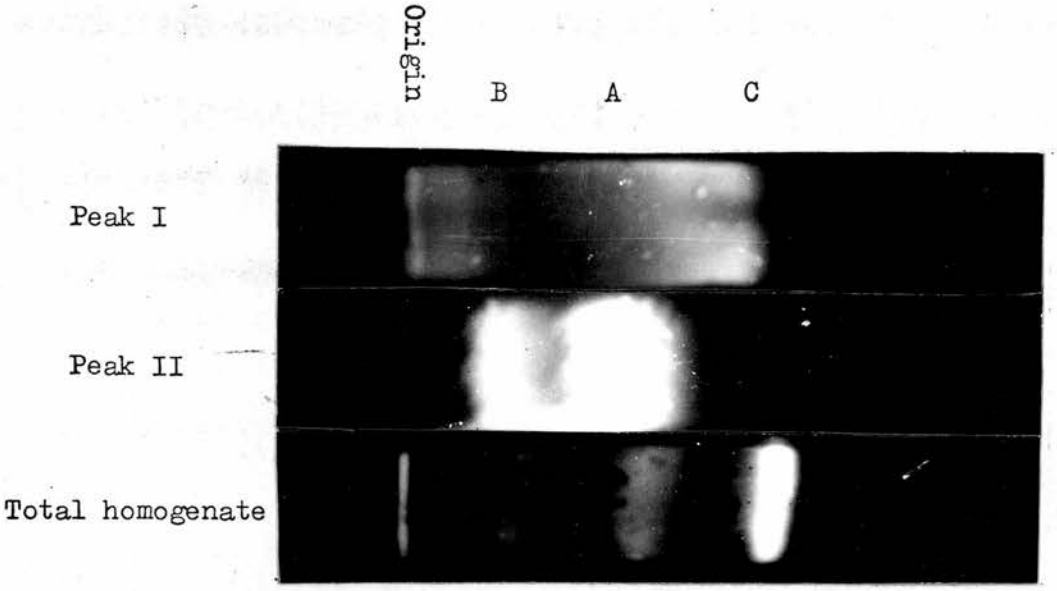


FIGURE 10. "Cellogel" electrophoresis of neonatal brain hexosaminidases showing peak I to be more anodic than peak II.

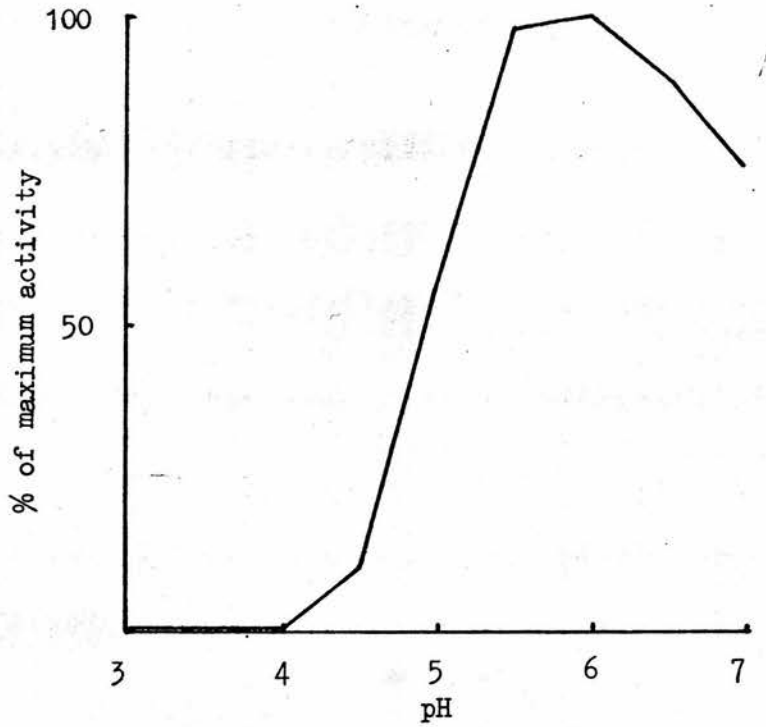


FIGURE 11. Activity (%) v. pH plot for peak I hexosaminidase from Sephadex G-200 gel filtration of neonatal brain.

assayed at pH 5.5 were 0.50mM and 0.81mM (Figure 12, page 40). Under the conditions used for the standard assay, the ratio of glucosaminidase activity to galactosaminidase activity was 31 for Hex-C.

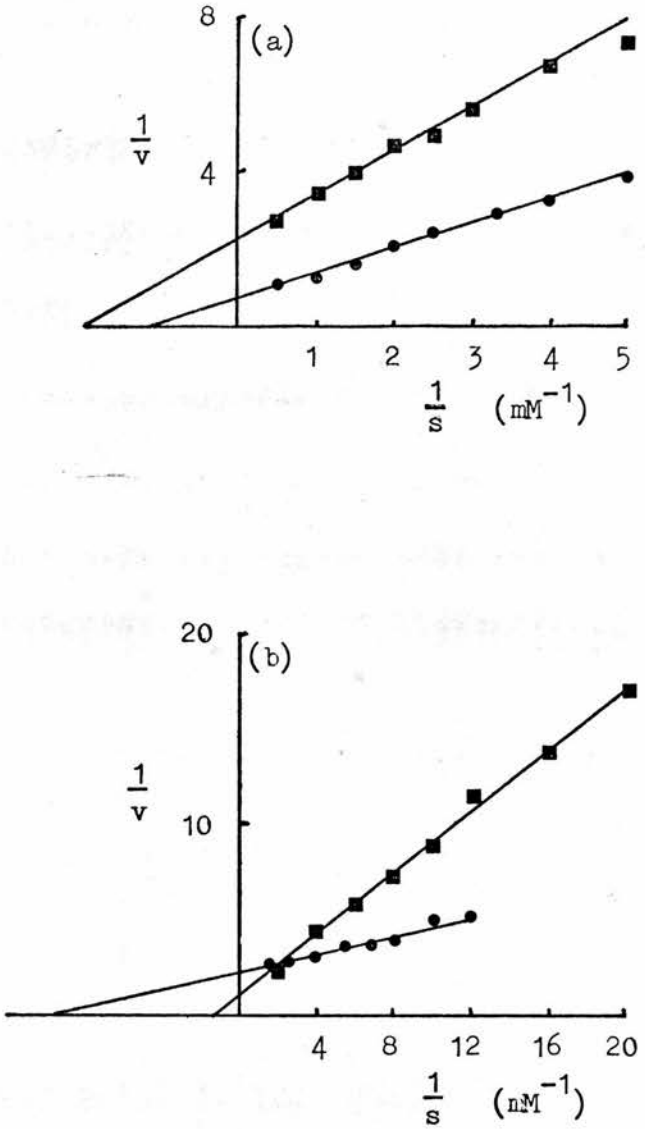


FIGURE 12. Lineweaver-Burk double reciprocal plots for the determination of apparent $K_m$. Peak I (■) was assayed at pH 5.5 whereas peak II (•) was assayed at pH 4.5.

a) Using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate. $K_m$ for peak I = 0.50mM and for peak II = 0.88mM.

b) Using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside as substrate. $K_m$ for peak I = 0.81mM and for peak II = 0.116mM.

(iv) Hexosaminidase S.

For the preparation of Hex-S, liver hexosaminidases were separated by DEAE-cellulose column chromatography (Figure 13).

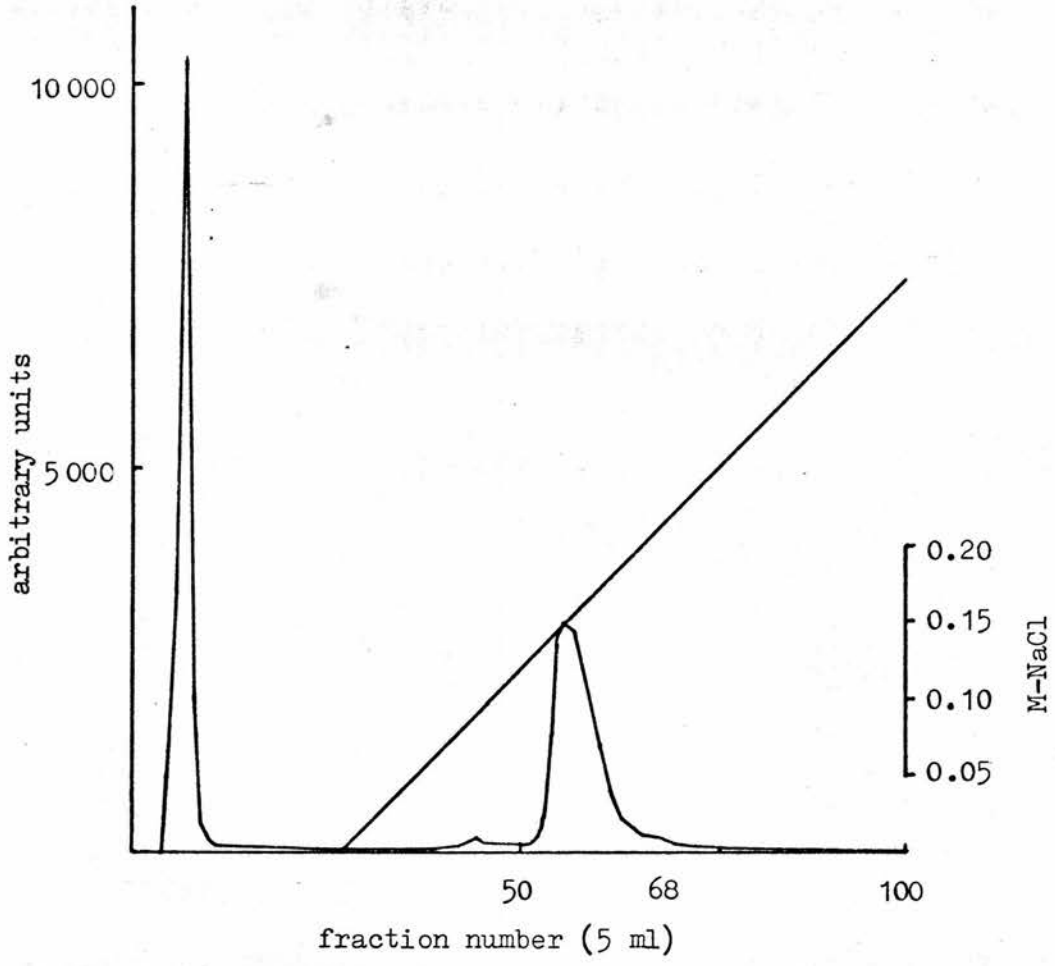


FIGURE 13. Separation of liver hexosaminidases with a view towards preparation of a fraction rich in hexosaminidase S by DEAE-cellulose column chromatography.

Fractions 68 and 69 were taken separately, dialysed against distilled water and used for focusing. The isoelectric focusing profile of fraction 68 (Figure 14) had three peaks of hexosaminidase activity with isoelectric points of pH 4.42, pH 5.02 and pH 7.43.

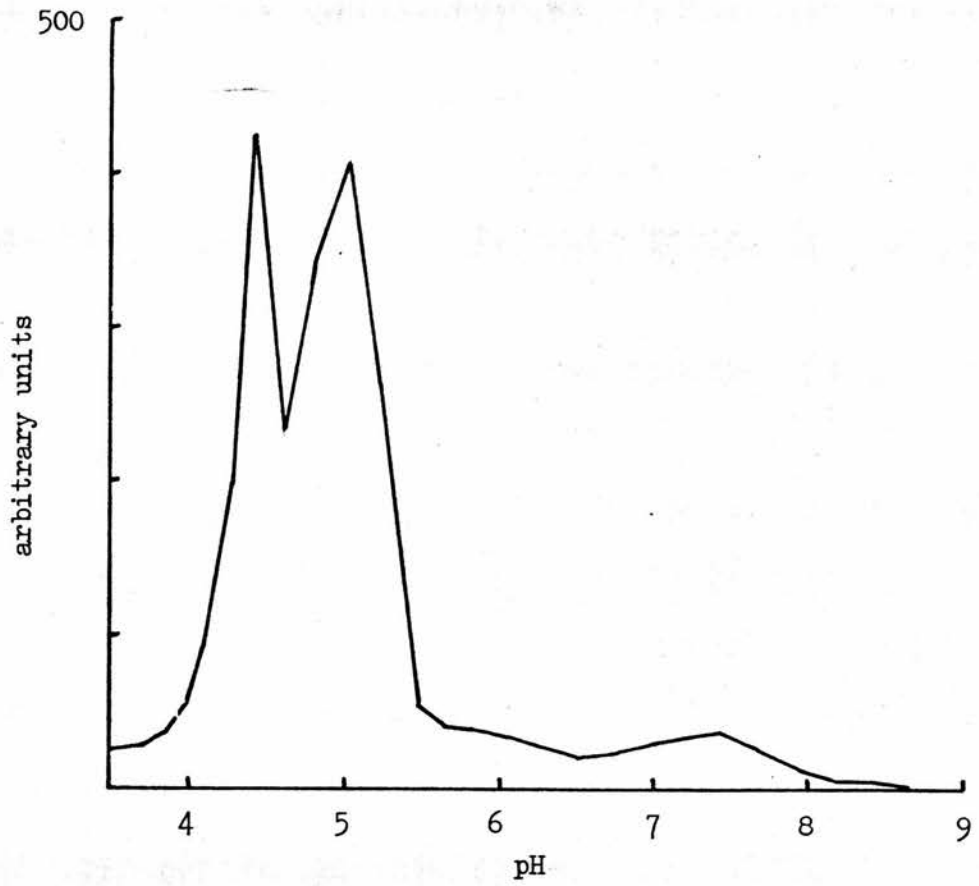


FIGURE 14. Isoelectric focusing of fraction 68 from the DEAE-cellulose column (Figure 13) in the range pH 3.5-10.0.

The fractions at pH 4.26 and pH 4.42 were pooled and the heat stability of hexosaminidase in these fractions compared with that of hexosaminidases A and B (Figure 15). Hex-A had a

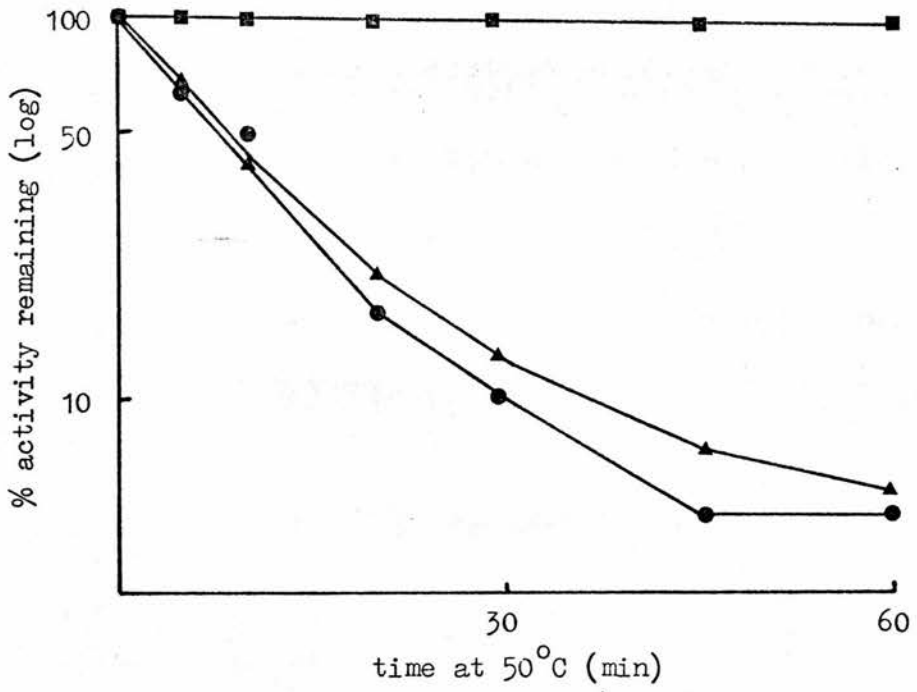


FIGURE 15. Heat inactivation plots of the peak corresponding to pH 4.42 in Figure 14 (▲) and hexosaminidases A (●) and B (■).

half life of about eight minutes, Hex-B was stable and the pooled fractions from the focusing column, believed to be mainly Hex-S, gave a biphasic plot. Most of this Hex-S peak (85%) was very heat labile with a half life of about eight minutes, but a minor component with a half life of about forty minutes was also present. In a pilot experiment, this minor component had been in excess of thirty percent of the activity. A further purification

step was considered to be necessary before meaningful $K_m$s could be obtained.

Accordingly, fraction 69 from the DEAE-cellulose column was focused in the range pH 4.0-6.0 and three peaks of activity at pH 4.43, pH 4.80 and pH 5.07 were obtained (Figure 16).

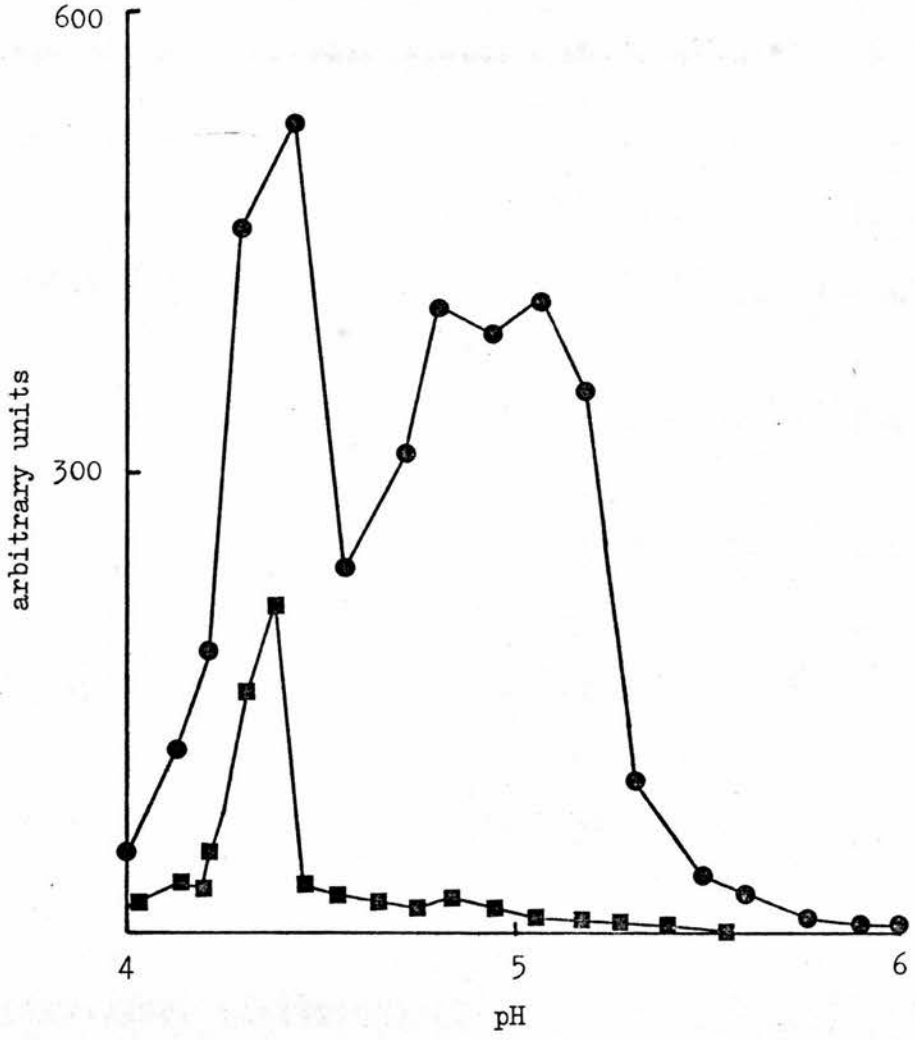


FIGURE 16. Isoelectric focusing of fraction 69 (Figure 13 ) in the range pH 4.0-6.0 (●) and refocusing of fractions corresponding to pH 4.00, 4.12, 4.21, 4.30 and 4.43 (■).

These latter two were probably hexosaminidase A of serum and tissue respectively. Fractions between pH 4.00 and pH 4.43 were dialysed against distilled water and refocused (Figure 16, page 44). The major peak of hexosaminidase had an isoelectric point of pH 4.38 and there were two smaller peaks at pH 4.13 and pH 4.84. The fractions at pH 4.38 and 4.31 were pooled and used for a pH profile and for the determination of the apparent $K_m$ with both glucosaminide and galactosaminide conjugates.

The pH profiles for hexosaminidases A, B and S are shown in Figure 17 (page 46). Hexosaminidases A and S both had optimal activity at pH 4.5, but the profile for component S was sharper especially on the neutral side. Hex-B had an optimum at pH 4.0-4.5.

The apparent $K_m$ at pH 4.0 was determined from the best straight line through a double reciprocal plot calculated by regression. The kinetics were studied at pH 4.0 because it was felt that subsequent studies on Sandhoff fibroblasts would have to take into account possible interference from Hex-C. The Lineweaver-Burk plots for hexosaminidases A, B and S are shown in Figure 18 (page 47). The apparent $K_m$ for Hex-S was found to be 10.5mM for glucosaminidase activity and 0.78mM for galactosaminidase activity. For Hex-A, the apparent $K_m$ was 2.4mM for glucosaminidase and 0.23mM for galactosaminidase, and for Hex-B 1.0mM and 0.124mM respectively.

Attempts to produce Hex-S from either hexosaminidase A or B failed. Using the method of Beutler and Kuhl (1975), Hex-A and Hex-B were each frozen and thawed three times in 20mM-sodium

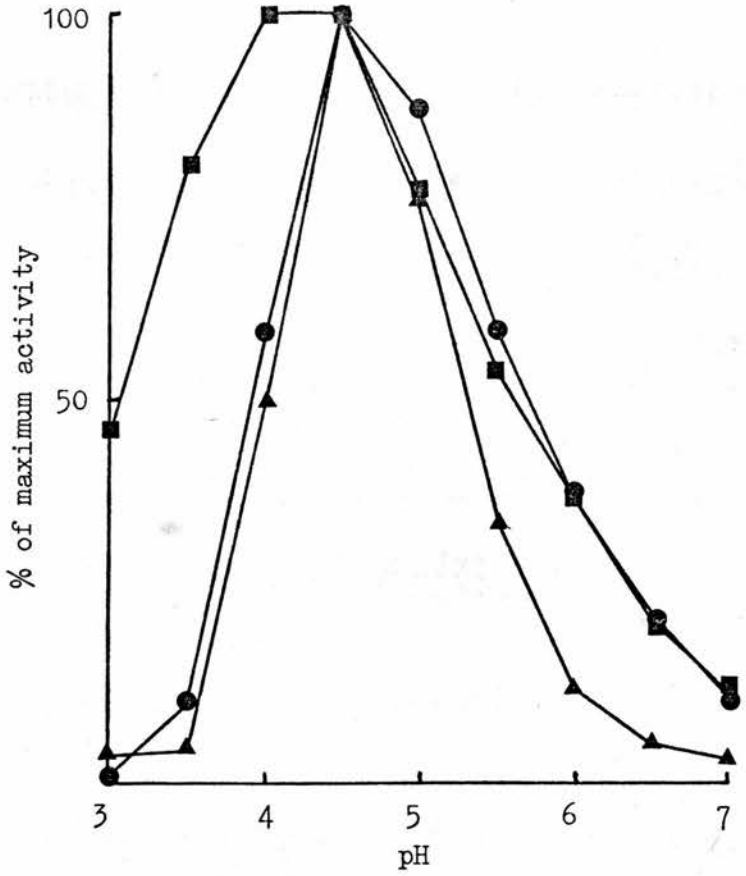


FIGURE 17. Activity (%) v. pH plots for hexosaminidases A (●), B (■) and S (▲).

phosphate buffer, pH 7.0, containing 3M-sodium chloride. In neither case was an extra peak of any significance produced (Figure 19, page 48). The conditions, however, were not quite as in the experiment described by Beutler and Kuhl (1975) in that the activity of the enzyme used was but a fifth of that recommended.

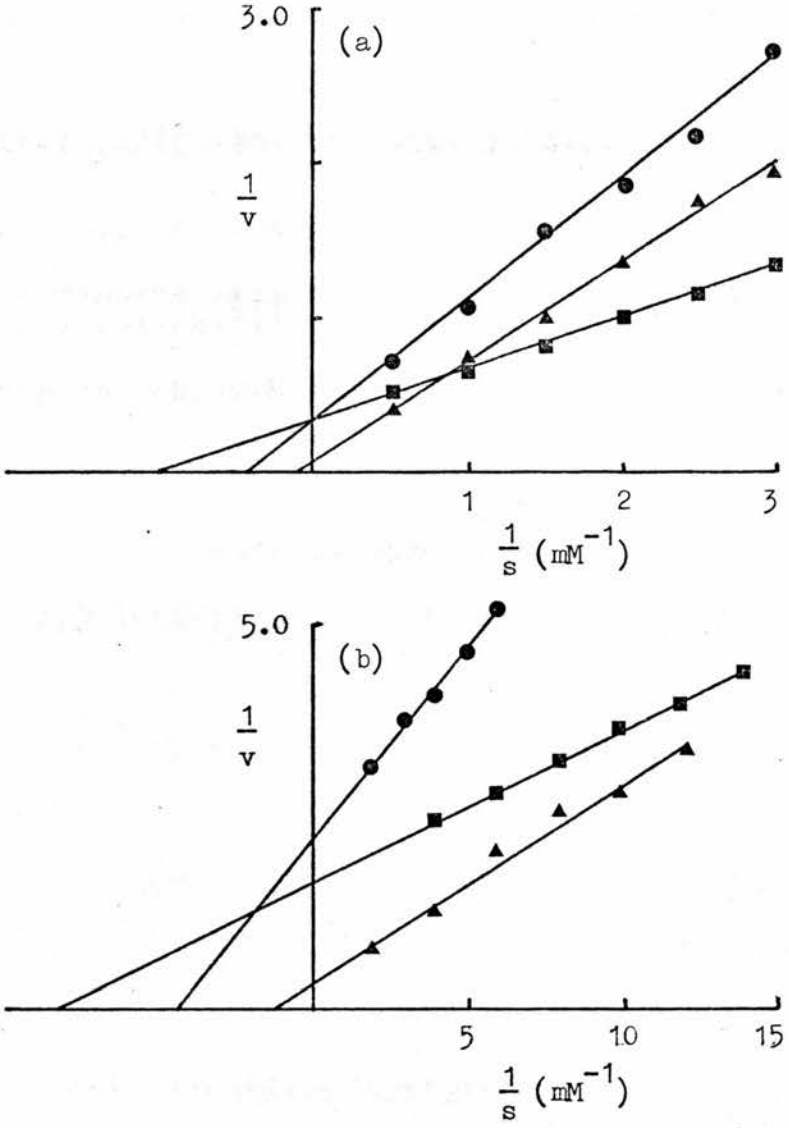


FIGURE 18. Lineweaver-Burk double reciprocal plots for the determination of apparent $K_m$ for hexosaminidases A (●), B (■) and S (▲) when assayed at pH 4.0.

a) Using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate. $K_m$ for A = 2.4mM, B = 1.0mM and S = 10.5mM.

b) Using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside as substrate. $K_m$ for A = 0.23mM, B = 0.124mM and S = 0.78mM.

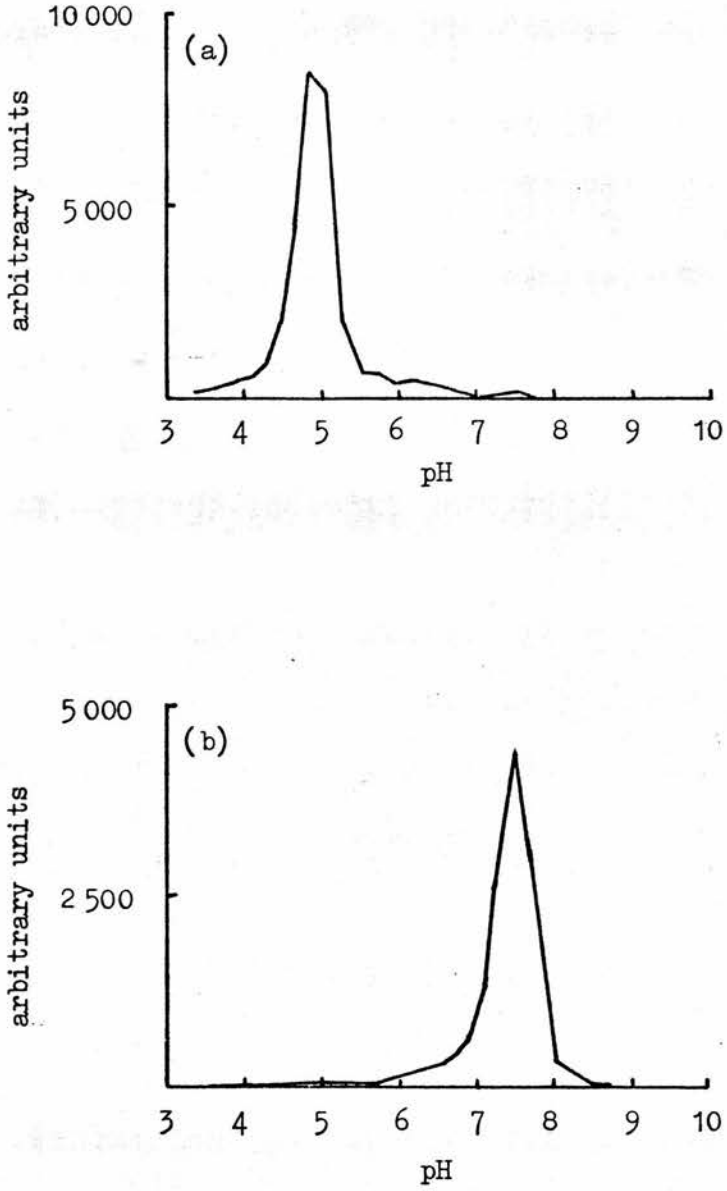


FIGURE 19. Isoelectric focusing of human liver hexosaminidases A (a) and B (b) in the range pH 3.5-10.0 after three cycles of freeze-thawing in 20mM-sodium phosphate buffer pH 7.0, containing 3M-sodium chloride.

DISCUSSION.

(i) Estimation of hexosaminidase A using DEAE-cellulose.

The two major peaks of hexosaminidase, components A and B, may be separated on DEAE-cellulose. This method was shown to be useful for the diagnosis of the $G_{M2}$ gangliosidoses (Young et al., 1970). The broad peak of hexosaminidase activity, eluting between components A and B, has been resolved into two peaks of activity (Price and Dance, 1972). These intermediate forms of hexosaminidase were found mainly in serum and were heat stable. They were named hexosaminidases $I_1$ and $I_2$ in order of their elution from DEAE-cellulose.

The DEAE-cellulose batch method was found to be particularly useful for determining the percentage of Hex-A in different types of cultured cells. This method gave more satisfactory results than the original heat inactivation method using commercial bovine serum albumin. Bovine serum albumin, which had been treated to remove hexosaminidase activity before use in the heat inactivation method, enabled an estimation of Hex-A activity, closely similar to that determined by the batch method. This improvement was not attributable to the removal of bovine hexosaminidase as better results were obtained than when distilled water was substituted. The DEAE-cellulose batch method was suitable for quick and simple analyses in laboratories where several determinations of percentage Hex-A were required but not sufficient to warrant an automatic system.

(ii) Percentage hexosaminidase A in different cell types.

It had been observed previously that cultured skin fibroblasts had a higher hexosaminidase activity than cultured amniotic fluid cells (Sutherland, 1974). In order to investigate the possible origin of amniotic fluid cells, Sutherland and Bain (1972) had attempted the culture of cells from the urine of new-born infants. Although the success rate was low, where growth was achieved, hexosaminidase levels were more like those of cultured amniotic fluid cells than skin fibroblasts. Unfortunately not enough urine cell strains were successfully cultured in order that the similarity to amniotic fluid cells and difference from skin fibroblasts could be shown statistically. Although, in this study, it became clear that the higher specific activities in fibroblasts were due to Hex-B and that cells cultured from amniotic fluid and urine consequently had a higher percentage Hex-A, once again insufficient urine cell strains were available for statistical differences to be shown.

(iii) Identification of hexosaminidase components by isoelectric focusing.

Isoelectric focusing was a more useful technique than ion exchange chromatography in that components were concentrated to a defined isoelectric point and that it was possible to look at all components at the same time if an ampholyte of sufficiently wide range was used. It was possible, therefore, to examine hexosaminidases A and B at the same time and to detect small differences between components. Hexosaminidases A and B in cultured cells were similar to those in liver, although liver was sometimes found to contain an appreciable amount of serum Hex-A, which had a slightly lower isoelectric point than the tissue form. Serum, apart from having a different Hex-A, had appreciable $I_1$ and $I_2$ activities. Pregnancy serum had large amounts of Hex-P, the isoelectric point of which corresponded to that of Hex-$I_2$. Previously, Stirling (1972) had suggested that Hex-P was identical to Hex-$I_2$ on the basis of its elution from DEAE-cellulose. Neonatal brain had considerable Hex-C activity, so much so that the combined peak of A and C was shifted to an intermediate isoelectric point. Assay at pH 4.5 and pH 5.5 showed that there were two components in this peak. There was no Hex-B in brain corresponding to that in liver and cultured cells. The broad peak of activity at pH 6.5-7.5 was probably a combination of $I_1$ and $I_2$. The situation in white blood cells was very complicated. The hexosaminidase activity, which would have shown up as component A using DEAE-cellulose, was resolved, at least partially, into five components. Polymorphonuclear cells appeared to have all five

components, whereas lymphocytes had three or maybe four. Two of these were probably tissue and serum Hex-A. It was more difficult to identify the other three components but peak I was possibly Hex-S.

(iv) Hexosaminidase C and its relevance to the $G_{M2}$ gangliosidoses.

Hex-C was just included in Sephadex G-200 and had an estimated molecular weight of around 190 000, in agreement with the findings of Penton et al. (1975). These workers, however, claimed that the isoelectric point of Hex-C was pH 5.7 not pH 4.80 as reported here. The molecular weight of hexosaminidases A and B was reported (Srivastava et al., 1974) to be 140 000 determined by gel filtration and 100 000 by the sedimentation equilibrium method, less than that of Hex-C.

Hex-C also differed from hexosaminidases A and B in its greater affinity for the glucosaminide conjugate of methylumbelliferone. As the function of components A and B would seem to be the removal of N-acetyl-galactosamine from $G_{M2}$ ganglioside, asialo $G_{M2}$ and globoside, it was not surprising that their apparent $K_m$ was significantly lower for the galactosaminide than for the glucosaminide. That peak I (Hex-C) had more affinity for the glucosaminide conjugate suggested that this particular enzyme was not primarily concerned with the cleavage of N-acetyl-galactosaminide linkages. That Hex-C preferred the glucosaminide substrate and had a more neutral pH optimum was recognised by other workers (Braidman et al., 1974; Penton et al., 1975). It seemed likely, therefore, that Hex-C was genetically unrelated to hexosaminidases A and B and was not the source of the unique subunit of Hex-A.

(v) Hexosaminidase S and its relationship to hexosaminidases A and B.

The peak of hexosaminidase activity corresponding to Hex-S had a lower isoelectric point than Hex-C. The half life of Hex-S was eight minutes, the same as that of Hex-A, under standard conditions for heat inactivation. Ikonne et al. (1975) quoted half lives of twenty two minutes and seventeen minutes for hexosaminidases A and S respectively. Even though the conditions used by these workers were slightly different, the heat stabilities of hexosaminidases A and S were very similar. The optimum activity of Hex-S was at pH 4.5, in agreement with Ikonne et al. (1975) and Beutler, Kuhl et al. (1975). This property alone suggested that component S was a more likely source of the unique subunit of Hex-A than Hex-C had been.

Hex-S had more affinity for the galactosaminide than for the glucosaminide conjugate but the apparent $K_m$s using both substrates were higher than those calculated for hexosaminidases A and B. Although Hex-S preferentially attacked the galactosaminide substrate, it was not nearly so active towards either substrate as hexosaminidases A and B. It has been reported (Beutler, Kuhl et al., 1975) that Hex-S, although precipitated by antiserum raised against Hex-A, required more antiserum per unit of enzyme activity than Hex-A. It was suggested that this was because Hex-S had less catalytic activity per molecule than Hex-A. The findings support the view that Hex-S is composed of the subunits differentiating Hex-A from Hex-B.

(b) STUDIES ON HEXOSAMINIDASES IN THE $G_{M2}$ GANGLIOSIDOSES.

RESULTS.

(i) pH profiles of the residual hexosaminidase of cultured skin fibroblasts from Sandhoff patients.

The hexosaminidase activity of fibroblasts from the two cases of Sandhoff's disease studied was less than 3% of the control mean. A pH profile of the residual hexosaminidase activity of Sandhoff fibroblasts is shown in Figure 20. With 4-MU-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate, the optimum was pH 5.5-6.0 in both cases, but there was a shoulder of activity at pH 4.0-4.5 and in this respect the profiles differed from that of Hex-C (Figure 11, page 39).

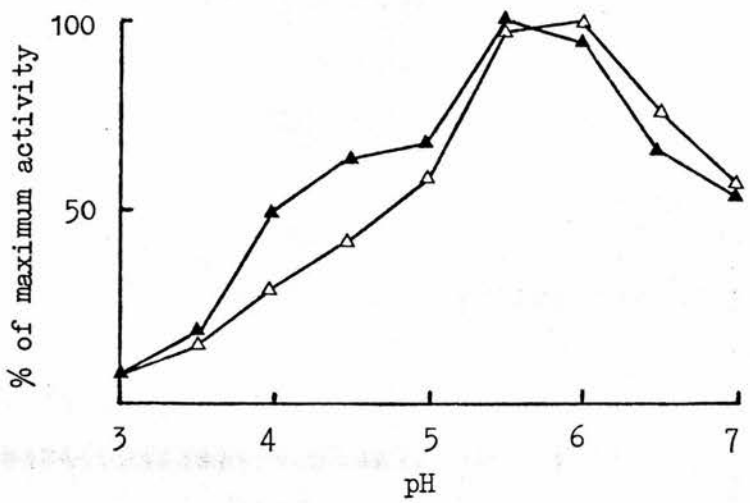


FIGURE 20. pH profiles using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) of cultured skin fibroblast homogenates from two cases of Sandhoff's disease.

case 1 (△) case 2 (▲)

The cells had been cultured in the presence of fetal bovine serum (15%$^{v}$/v) and it was possible that the shoulder of activity at pH 4.0-4.5 was attributable to bovine serum hexosaminidase taken up by the cells. To establish whether this was indeed the case, a culture of Sandhoff fibroblasts was subcultured into two culture vessels, one of which was treated in the usual way and the other, using medium supplemented with heat inactivated fetal bovine serum, which had been shown to have no hexosaminidase activity. The cells grown on inactivated serum had a reduced shoulder at pH 4.0-4.5 compared with the control experiment, but the profile still did not correspond to Hex-C (Figure 21). It was concluded that at least some of the activity in the acid range was intracellular in origin.

If 4-MU-2-acetamido-2-deoxy-β-D-galactopyranoside was the

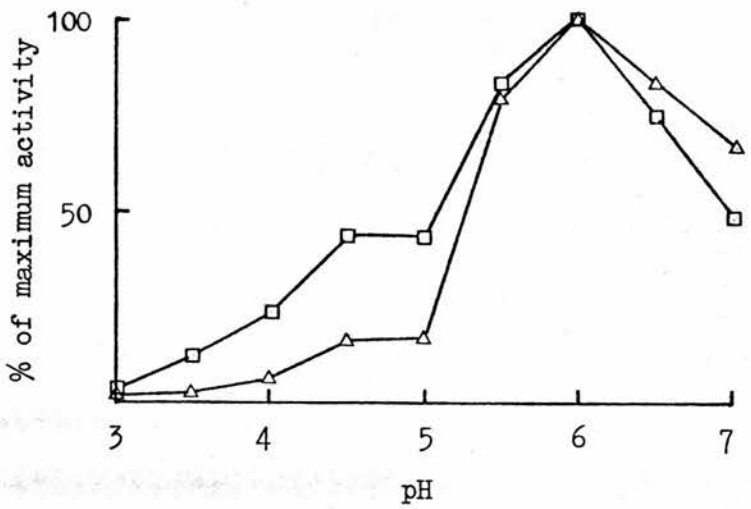


FIGURE 21. Comparison of pH profiles using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) of Sandhoff (case 2) fibroblasts cultured on heat inactivated (△) and untreated serum (□).

substrate, the activity at pH 4.5 relative to that at pH 5.5 was greatly increased (Figure 22). The components with optima at

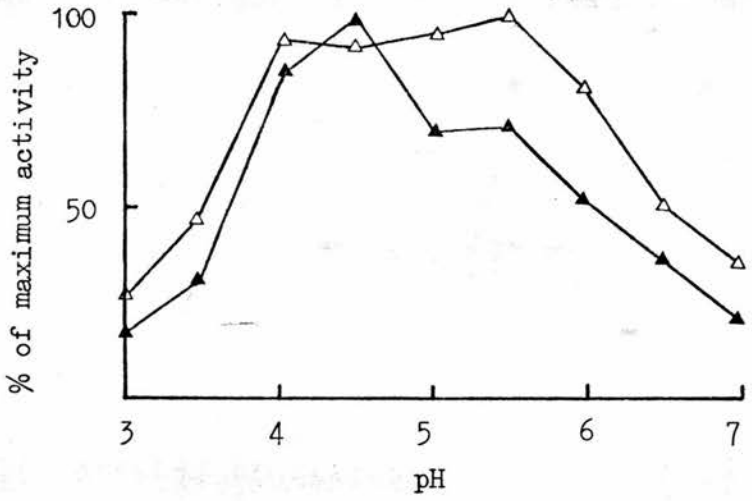


FIGURE 22. pH profiles using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (0.5mM) of cultured skin fibroblast homogenates from two cases of Sandhoff's disease.
case 1 (△) case 2 (▲)

pH 4.5 and pH 5.5 differed considerably in their relative activities to the two substrates. When Sandhoff fibroblasts grown on heat inactivated serum were compared with those grown on normal serum, there was still activity at pH 4.5 using the galactosaminide conjugate as substrate (Figure 23, page 58). The glucosaminidase activity at pH 4.5 was reduced by 61% when Sandhoff cells were cultured using heat inactivated serum and the galactosaminidase activity was reduced by 51%. The increase in activity at pH 4.5 relative to that at pH 5.5 when using the galactosaminide conjugate was not, therefore, wholly attributable to hexosaminidase taken up by the serum. The nature of the residual intracellular enzyme, active at pH 4.5, was investigated further.

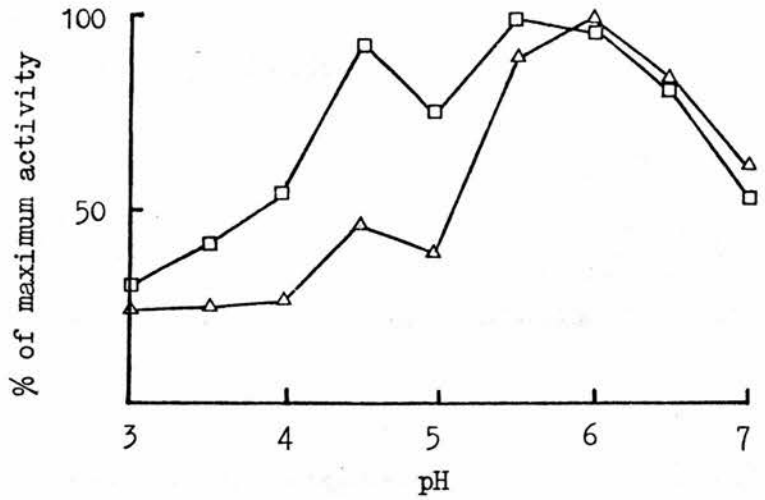


FIGURE 23. Comparison of pH profiles using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (0.5mM) of Sandhoff (case 2) fibroblasts cultured on heat inactivated (△) and untreated serum (□).

(ii) Isoelectric focusing of Sandhoff fibroblast homogenates.

There were two major peaks of hexosaminidase activity when Sandhoff fibroblast homogenates were focused (Figure 24, page 60). Control fibroblast homogenates, focused at the same time, had two much larger peaks of activity, corresponding to hexosaminidases A and B, with isoelectric points at pH 5.5 and pH 8.2 respectively. In later experiments, the isoelectric points of hexosaminidases A and B were somewhat lower. It was not certain what caused this shift, but it must be taken into account when discussing isoelectric points. For Sandhoff fibroblasts, the peak corresponding to Hex-B was broader than for the control and much reduced. The other peak in Sandhoff fibroblasts had an isoelectric point of pH 4.80, when assayed at pH 5.5, the pH optimum of Hex-C (see page 39). When assayed at pH 4.5, the isoelectric point was pH 4.65.

Sandhoff fibroblasts, cultured on heat inactivated serum differed from those cultured on normal serum with respect to their isoelectric focusing profile (Figure25, page 61 ). The major difference was in the Hex-B peak. Cells cultured on inactivated serum had a sharp peak of Hex-B at pH 7.94 whereas, with cells cultured under control conditions, there was a broad peak of activity at pH 7.15-7.84, as had been found previously (page 60 ). Under both conditions of culture, there was a recognisable peak of Hex-A at pH 5.22. This had been observed earlier for this cell strain (case II). The other cells (case I) studied had no detectable Hex-A, even when cultured on untreated fetal bovine serum. Although both cases had similar clinical courses, there did seem

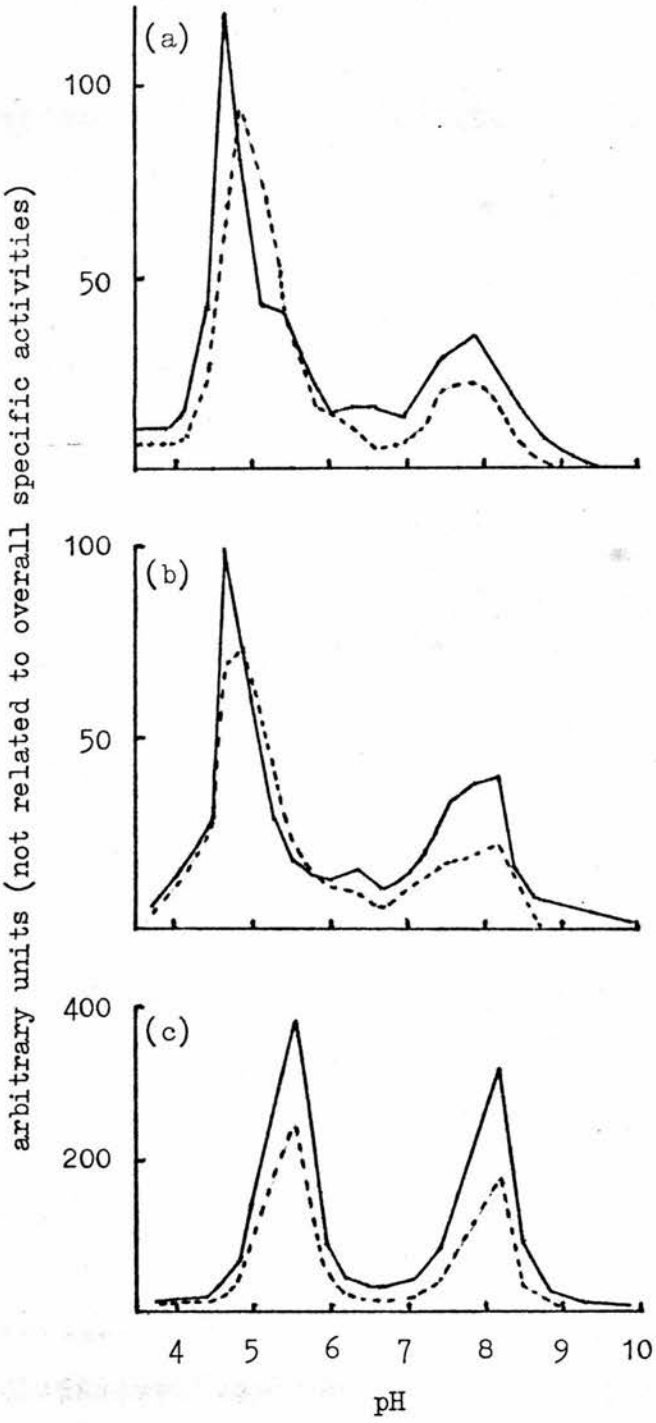


FIGURE 24. Isoelectric focusing in the range pH 3.5-10.0 of fibroblast extracts from (a) Sandhoff case 2, (b) Sandhoff case 1 and (c) control. Assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) at pH 4.5 ——— and pH 5.5 - - - -.

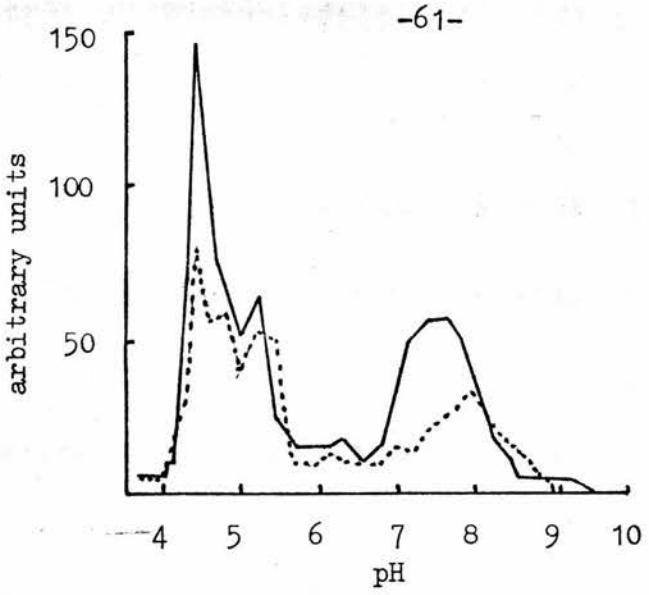


FIGURE 25. Isoelectric focusing in the range pH 3.5-10.0 of Sandhoff (case 2) fibroblast extracts from cells cultured using untreated serum ——— and heat inactivated serum - - - - -. Fractions were assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) at pH 4.5.

to be slight differences in the residual hexosaminidases of these two unrelated patients. The major anodic peak under both culture conditions had an isoelectric point of pH 4.41 corresponding to pH 4.65 in earlier experiments.

(iii)Changes in Sandhoff fibroblast hexosaminidases on storage.

The apparent shift in the optimum of the major residual hexosaminidase of Sandhoff fibroblasts from pH 5.5-6.0 to pH 4.5, together with a lowering of isoelectric point was investigated. The activity of the hexosaminidase of Sandhoff fibroblasts at pH 4.5 increased relative to that at pH 5.5 after dialysis overnight at 4°C against distilled water (Figure 26 , page 63 ), but this did not occur to the same extent if the extract was dialysed against 10mM-sodium acetate buffer, pH 5.5. Furthermore, it became apparent that there was no actual increase in hexosaminidase activity at pH 4.5, but that the change in pH profile was attributable to a decrease in the activity at pH 5.5-6.0.

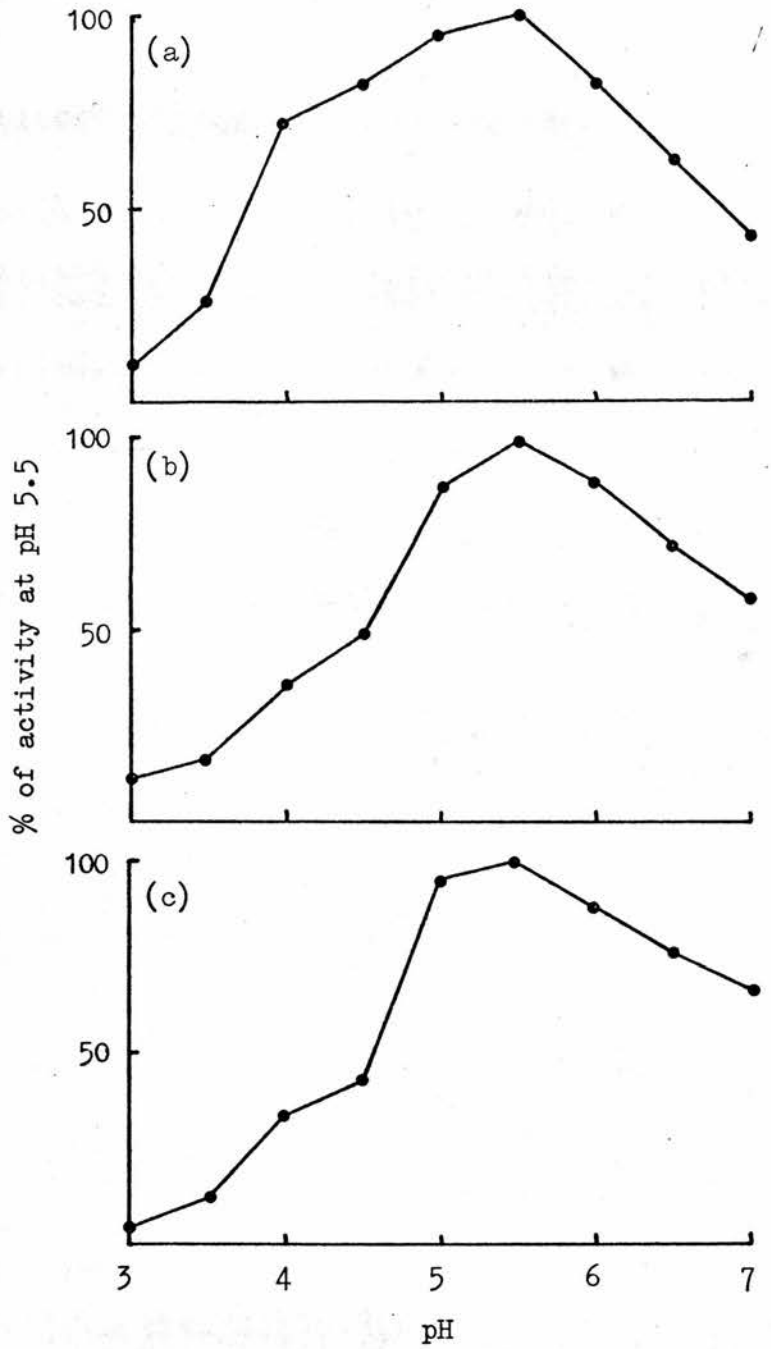


FIGURE 26. Effect on the N-acetyl-β-D-glucosaminidase pH profile of Sandhoff (case 1) fibroblasts when dialysed overnight at 4°C against (a) distilled water, (b) 10mM-sodium acetate buffer, pH 5.5, compared with (c) extract stored at 4°C overnight.

(iv) Separation of the hexosaminidases of extracts of $G_{M2}$ gangliosidosis fibroblasts by electrophoresis.

Fibroblast hexosaminidases from control and $G_{M2}$ gangliosidosis patients were separated by electrophoresis on "Cellogel" and visualised by assay at pH 4.5 and pH 5.5 (Figure 27, page 65 ). Control fibroblast extracts had three bands of enzyme activity, the fastest of which was low in activity. Sandhoff fibroblasts had only one band of activity, corresponding to the fastest moving band in control fibroblasts and, although this was not understood at the time, seemed to have more activity at pH 4.5 than pH 5.5. This also corresponded in mobility to Hex-C, prepared from neonatal brain. Tay-Sachs fibroblasts had two bands of hexosaminidase activity, corresponding in position to components B and C.

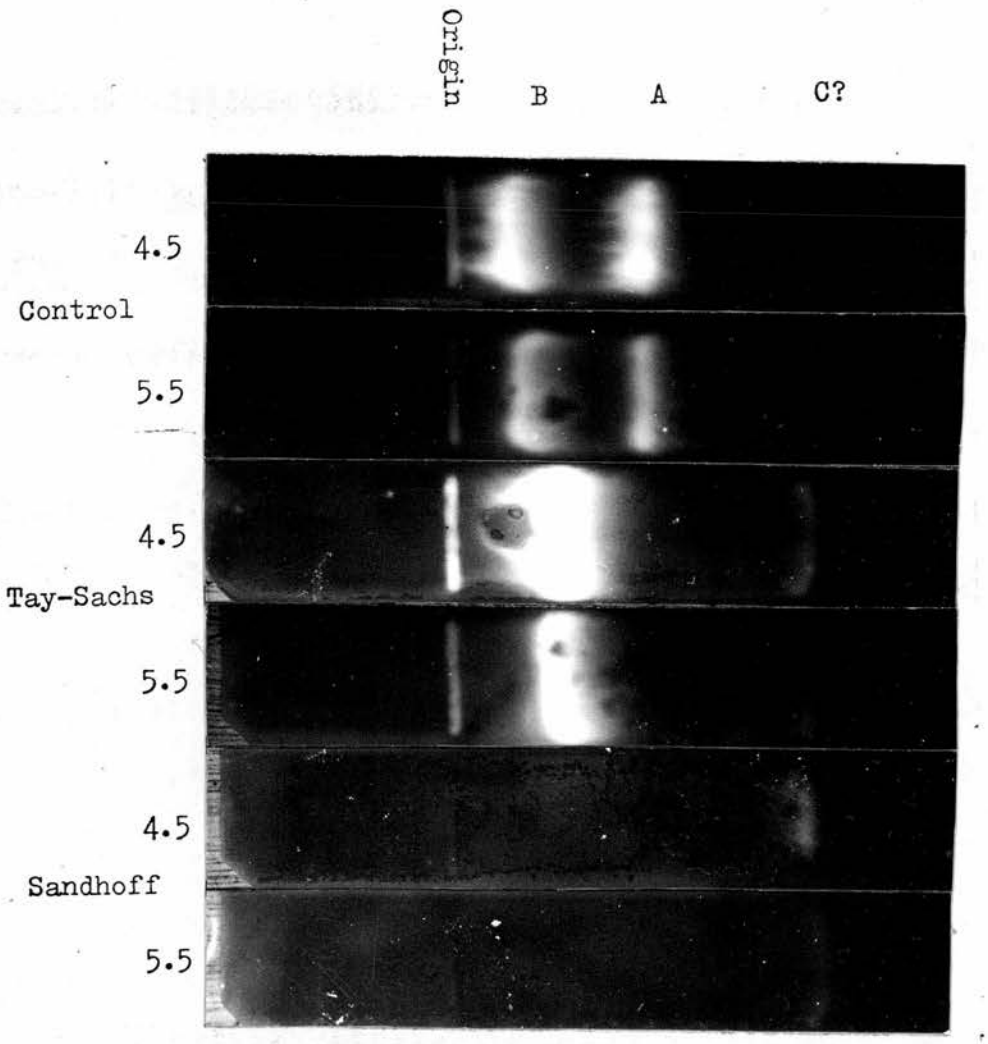


FIGURE 27. "Cellogel" electrophoresis of fibroblasts hexosaminidases from control, Tay-Sachs and Sandhoff patients, visualised by assay at pH 4.5 and pH 5.5.

(v) The residual hexosaminidase in Sandhoff leucocytes.

The pH profile of the hexosaminidase extracted from Sandhoff leucocytes (case II) had an optimum of pH 4.5, which was the same as control extracts, but the peak was much sharper on the acid

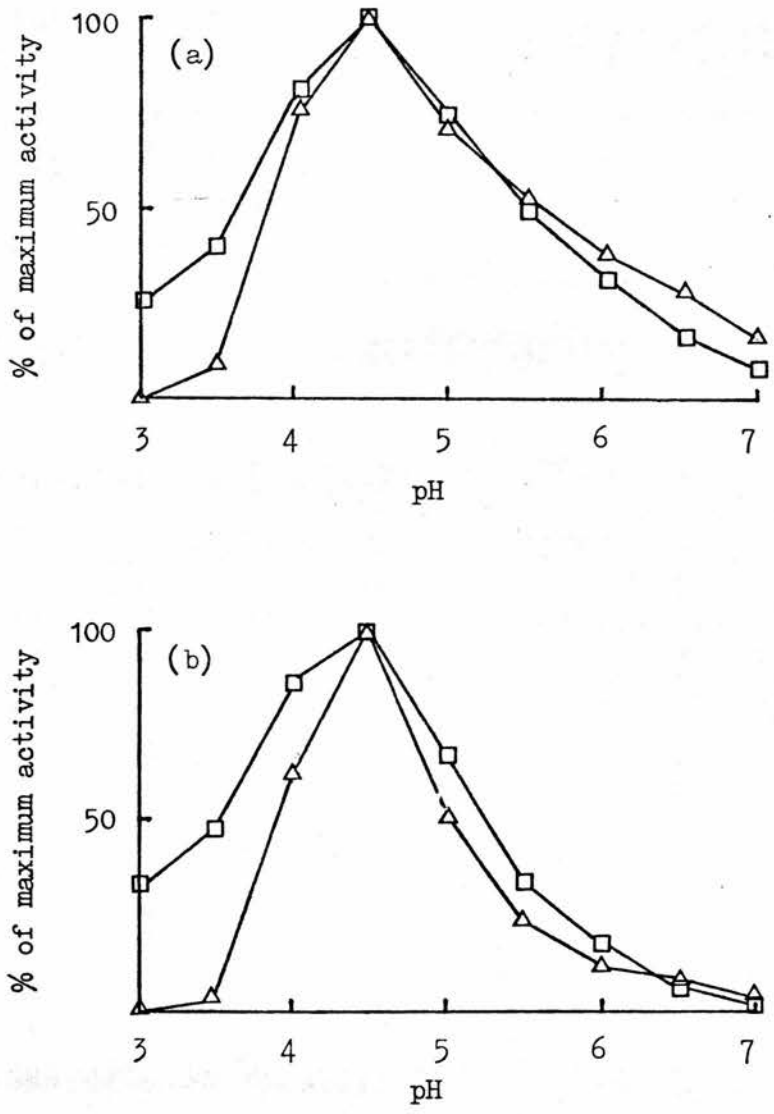


FIGURE 28. pH profiles of Sandhoff (case 2) (△) and control (□) leucocytes using:-

(a) 4-MU-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM).

(b) 4-MU-2-acetamido-2-deoxy-β-D-galactopyranoside (0.5mM).

side. The profiles differed slightly depending on whether the glucosaminide or the galactosaminide conjugate was used. With the glucosaminide conjugate, the control extract had a sharper drop in activity on the neutral side (Figure 28 a, page 66 ), whereas with the galactosaminide conjugate, the Sandhoff extract had the sharper optimum (Figure 28 b, page 66 ).

The nature of the residual hexosaminidase in Sandhoff leucocytes was also studied by isoelectric focusing. Using control leucocytes, earlier studies had indicated the presence of a number of hexosaminidase components with isoelectric points around pH 5.0 (page 34). In this experiment, hexosaminidases were focused in the range pH 3.5-10.0 and so resolution of these anodic components was not expected (Figure 29). Sandhoff leucocytes had a small amount of Hex-B, but the major components had

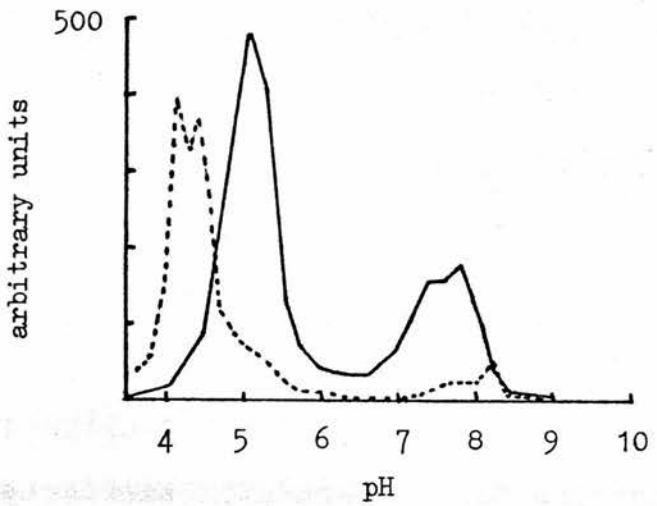


FIGURE 29. Isoelectric focusing in the range pH 3.5-10.0 of control ——— and Sandhoff (case 2) ---- leucocytes assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM) at pH 4.5.

isoelectric points of pH 4.41 and pH 4.16. These were not detectable in the control, due to the presence of Hex-A. There was only a small shoulder of Hex-A activity in Sandhoff leucocytes (case II). Refocusing of the major Sandhoff peaks in the range pH 4.0-6.0 gave the two peaks once again, with isoelectric points pH 4.41 and pH 4.15 respectively, confirming that the latter was indeed a different hexosaminidase.

(vi) Diagnosis of Tay-Sachs and Sandhoff's disease.

Unlike Sandhoff's disease, in which the enzyme deficiency may be recognised simply by assay of the total hexosaminidase activity, Tay-Sachs disease is diagnosed by the assay of hexosaminidase component A. Diagnosis may be achieved by a variety of methods, examples of which are DEAE-cellulose chromatography, isoelectric focusing, heat inactivation (Figure 30 , page 70 ) and electrophoresis (Figure 27, page 65 ). It was also noted that the hexosaminidase pH profile of Tay-Sachs extracts had a flatter optimum (pH 4.0-4.5) than that of controls with relatively higher activity at pH 3.0-4.0 (Figure 31, page 71 ).

Diagnosis of Tay-Sachs and Sandhoff's diseases was usually undertaken in the first instance on leucocytes as part of a screen for lysosomal enzyme deficiencies. If a positive result was found, this was confirmed on serum and cultured skin fibroblasts. Table 3 (page 72 ) shows hexosaminidase activities for controls and for cases and carriers of the $G_{M2}$ gangliosidoses. The percentage Hex-A was calculated from results, obtained using the DEAE-cellulose batch method. Case II of Sandhoff's disease had hexosaminidase activity which was 8% of the control mean in leucocytes and 3% for serum. Almost all the activity in leucocytes appeared to be Hex-A or some other component of hexosaminidase which was bound to DEAE-cellulose at 50mM-NaCl but unbound at 250mM-NaCl. The percentage Hex-A in Sandhoff serum also appeared to be higher than in controls.

The Sandhoff heterozygotes tested did not have particularly low levels of hexosaminidase in leucocytes, but the activity in

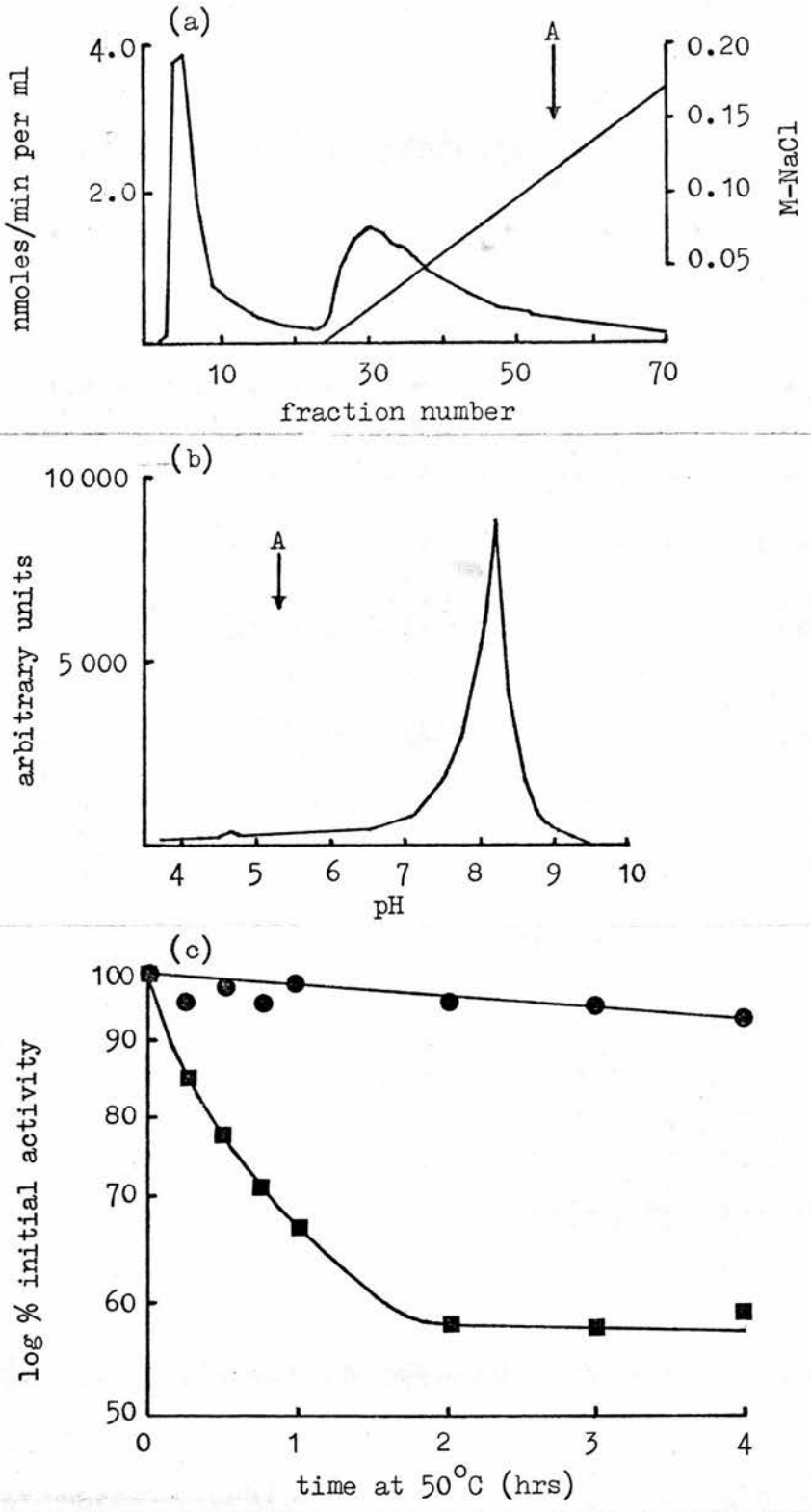


FIGURE 30. Diagnosis of Tay-Sachs disease using cultured skin fibroblasts by (a) DEAE-cellulose separation of components A and B, (b) isoelectric focusing and (c) heat inactivation ( Tay-Sachs (●), control (■)).

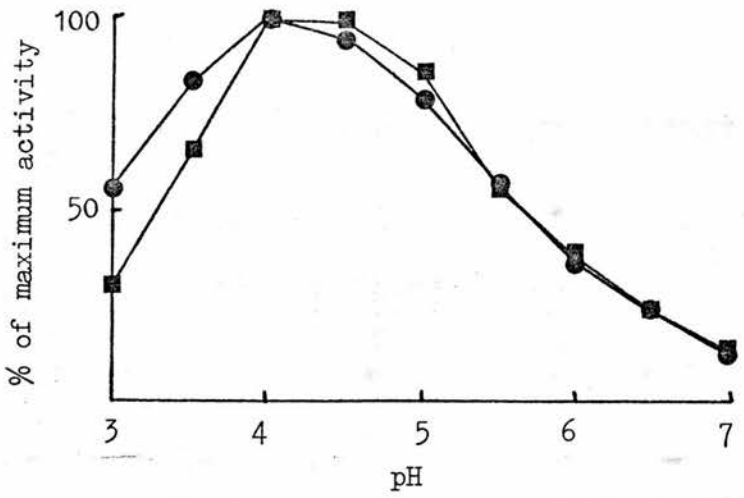


FIGURE 31. pH profile of fibroblast extracts from a case of Tay-Sachs disease (●) and a control (■) assayed using 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2mM).

serum was reduced to about a third of the mean control value. The percentage Hex-A in both leucocytes and serum of the Sandhoff heterozygotes was above the control range.

The hexosaminidase activity of Tay-Sachs leucocytes, although below the control mean, was within the control range. The activity in Tay-Sachs serum was just below the control range. It was not possible, however, to distinguish the Tay-Sachs from controls by assay of total hexosaminidase in leucocytes or serum with any certainty. The percentage Hex-A in Tay-Sachs leucocytes and serum was 9% and 2% respectively.

The total hexosaminidase level in leucocytes and serum from a Tay-Sachs heterozygote was again lower than the control mean, but well within the control range. Leucocytes from a heterozygote had 60% Hex-A, well within the control range, but serum from the same person had only 34% Hex-A, which was below the control range.

TABLE 3 Hexosaminidase activities in leucocytes and serum of controls and of carriers and cases of $G_{M2}$ gangliosidosis.

LEUCOCYTES.

| | | nmoles/min per mg protein | %A |
|---|---|---|---|
| Controls | mean | 32.6 | 66 |
| n = 80 | range | 9.6-63.5 | 50-76 |
| Sandhoff | | 2.65 | 98 |
| Tay-Sachs | | 19.1 | 9 |
| Sandhoff carriers | 1 | 17.1 | 85 |
| | 2 | 28.9 | 77 |
| Tay-Sachs carrier | | 25.4 | 60 |

SERA.

| | | nmoles/min per ml | %A |
|---|---|---|---|
| Controls | mean | 22.9 | 60 |
| n = 9 | range | 17.4-41.6 | 45-70 |
| Sandhoff | | 0.63 | 87 |
| Tay-Sachs | | 13.5 | 2 |
| Sandhoff carriers | 1 | 7.6 | 79 |
| | 2 | 7.7 | 79 |
| Tay-Sachs carrier | | 19.0 | 34 |

(vii) Antenatal diagnosis for Sandhoff's disease.

During the course of the study, antenatal diagnosis for Sandhoff's disease was undertaken on two occasions. The two families involved were those on whom studies were carried out as described in this section.

The results for each family are set out in Table 4 (page 74 ). Diagnoses were possible using the amniotic fluid supernatant for this disease, but it is recommended that assays should also be carried out on cultured amniotic fluid cells. In each case, hexosaminidase activity was below control levels, but well above that of cultured skin fibroblasts from the proband. The importance of assaying controls cultured at the same time is obvious from the results, since control levels differed on the two occasions. This could have been due to unexplained culture variables.

TABLE 4 Antenatal diagnosis for Sandhoff's disease.

| | Family 1 | Family 2 |
|---|---|---|
| Amniotic fluid | Hexosaminidase activity (nmoles/min per ml) | |
| Test | 3.06 | 5.01 |
| Controls | 5-20 | 11.1 |
| Cultured amniotic fluid cells | Hexosaminidase activity (nmoles/min per mg protein) | |
| Test | 31.4 | 16.4 |
| Controls | 57.8<br>51.5<br>43.7 | 28.3<br>30.6<br>33.7 |
| Fibroblasts from proband | 1.0 | 0.9 |
| Mother's fibroblasts | - | 14.7 |

Results for amniotic fluid were regarded as preliminary and diagnosis of fetuses probably heterozygous for Sandhoff's disease was based largely on the results for amniotic fluid cells.

DISCUSSION.

(i) The residual hexosaminidase in Sandhoff fibroblasts.

The major residual hexosaminidase in Sandhoff fibroblasts was Hex-C. Apart from being more anodic than Hex-A on "Cellogel" electrophoresis, it also corresponded to Hex-C prepared from neonatal brain in its neutral pH optimum, its greater affinity for the glucosaminide conjugate of 4-methylumbelliferone and its isoelectric point.

Hex-C was not, however, the only residual hexosaminidase in Sandhoff fibroblasts. A shoulder of activity at pH 4.0-4.5 was present, which was not seen in the pH profile of Hex-C, and it was possible that this was fetal bovine hexosaminidase, taken up from the tissue culture medium. Some of the activity at pH 4.0-4.5 was indeed shown to be due to contamination by exogenous hexosaminidases, but activity was present even in cells cultured in medium supplemented with heat inactivated serum, deficient in hexosaminidase activity.

The residual activity in the acid range differed from Hex-C in its relative activities towards the glucosaminide and galactosaminide conjugates of 4-methylumbelliferone and as with hexosaminidases A and B had more affinity for the galactosaminide substrate. Isoelectric focusing of Sandhoff fibroblasts revealed the presence of a small broad band of Hex-B and a major peak with pI 4.8 when assayed at pH 5.5, and pI 4.65 when assayed at pH 4.5. At first this was difficult to explain, especially since the peak with maximum activity at pH 4.5 was now the major component.

When the hexosaminidases of Sandhoff fibroblasts cultured using inactivated serum were analysed, the peak of activity corresponding to Hex-B was much sharper. Although other hexosaminidases may have been taken up by the cells, the most obvious one was Hex-$I_1$. The isoelectric point of the acid hexosaminidase in Sandhoff fibroblasts under either condition of culture was now pH 4.41.

The change of pH optimum of the residual hexosaminidase in Sandhoff fibroblasts could be explained by the lability of Hex-C. This was hastened by dialysis against distilled water or during isoelectric focusing. Dialysis against 10mM-sodium acetate buffer, pH 5.5, resulted in very little loss of activity, indicating that Hex-C was particularly labile at low ionic strengths. Reports that Hex-C is not the major residual hexosaminidase component in Sandhoff's disease (Ikonne et al., 1975; Beutler, Kuhl et al., 1975) might be explained by the lability of this component.

(ii) Hexosaminidase S in Sandhoff's disease.

The hexosaminidase of Sandhoff leucocytes differed from that of cultured skin fibroblasts. In leucocytes there was little evidence of the presence of Hex-C, except the slightly greater relative activity on the neutral side when the enzyme was assayed using the glucosaminide rather than the galactosaminide substrate. The Hex-C could have been lost during preparation. Isoelectric focusing showed that Sandhoff leucocytes had traces of Hex-B, but that most of the enzyme was in two peaks of activity with isoelectric points of pH 4.41 and pH 4.16.

Hex-S, prepared from control human liver, had an isoelectric point of pH 4.38, which corresponded to the major residual acid hexosaminidase in Sandhoff fibroblasts and one of the two major residual hexosaminidases in Sandhoff leucocytes. Wood and MacDougal (1976) also reported that the major residual hexosaminidase in fibroblasts from a case of infantile Sandhoff's disease had an isoelectric point of pH 4.40 and that there was very little of components A and B. A case of juvenile Sandhoff's disease, also reported in the same paper, had more residual Hex-A than Hex-S, with Hex-B activity again very low. There did seem to be some difference in the two cases reported in this thesis. Case I had a lower overall activity and was apparently totally deficient in Hex-A, whereas case II had a small shoulder of Hex-A activity even when cultured in medium supplemented with heat inactivated serum.

It has been reported (Beutler, Kuhl et al., 1975) that there are two forms of Hex-S in Sandhoff fibroblasts separable on DEAE-

cellulose, the major component eluting second. The minor component, Hex-S', was eluted from DEAE-cellulose at about the same chloride concentration as Hex-A, but was shown to differ from Hex-A as it was not precipitated by antibody raised against Hex-B. Ikonne et al., (1975), also using DEAE-cellulose, found two peaks of hexosaminidase activity in Sandhoff liver. These peaks were of similar activity and the first corresponded in position to Hex-A. Similar findings were also reported for plasma. The peak attributed to Hex-A in Sandhoff liver was probably Hex-S'. The twin peaks of activity on isoelectric focusing of Sandhoff leucocyte hexosaminidases, reported in this thesis, were probably the two hexosaminidase S components. Only one of these components was detected in Sandhoff fibroblasts with an isoelectric point of pH 4.41 and it was likely that the major component obscured the minor. If the double focusing peak in Sandhoff leucocytes corresponded to that in Sandhoff liver using DEAE-cellulose, the enzyme component with pI 4.16 was Hex-S'. From the DEAE-cellulose work, it might have been predicted that the isoelectric point of Hex-S' was on the neutral side of Hex-S. This does not have to be the case, however, as previously it has been shown that serum Hex-A is more anodic (Swallow et al., 1974) and has a lower isoelectric point (Besley and Broadhead, 1976; see page 31) than tissue Hex-A, but is eluted from DEAE-cellulose before the tissue enzyme (Ikonne and Ellis, 1973). It is possible that Hex-S' contains a subunit of serum Hex-A, whereas Hex-S contains a subunit of the tissue enzyme. Beutler and Kuhl (1975) suggested that Hex-S was more anodic than Hex-S', but there was no evidence

that electrophoresis was on peaks separated on DEAE-cellulose. Two very close bands of activity were resolved and these were assigned Hex-S and Hex-S' on the basis of the order of elution of these components from DEAE-cellulose.

(iii)Hexosaminidase A in the $G_{M2}$ gangliosidoses.

The difference in hexosaminidase pH profile between Tay-Sachs and controls could be predicted from the different profiles of hexosaminidases A and B. This might be explained by the difference in stability of hexosaminidases A and B particularly at acid pH (Saifer and Rosenthal, 1973), but this difference was only found for a critical pH range around pH 2.8.

The level of hexosaminidase activity in heterozygotes for the $G_{M2}$ gangliosidoses was dependent on the enzyme source. The total hexosaminidase activity was of no value in recognising Tay-Sachs heterozygotes or homozygotes. It had previously been reported that the total hexosaminidase activity was raised in Tay-Sachs brain but not in liver and kidney (Sandhoff et al., 1968; Okada and O'Brien, 1969). The hexosaminidase activity was not found to be raised in Tay-Sachs leucocytes and the serum level was slightly lower than controls. The hexosaminidase activity of Sandhoff leucocytes and serum was markedly deficient and well separated from the lowest control value. Whilst the leucocyte activities of Sandhoff heterozygotes were below the mean control value, they were nevertheless in the control range. However, when serum was used, heterozygous hexosaminidase levels were well below the control range.

The percentage Hex-A for patients and heterozygotes was particularly interesting. Very low percentage Hex-A was diagnostic of Tay-Sachs disease in leucocytes and serum. It was possible to distinguish the Tay-Sachs heterozygote from controls by measuring the percentage Hex-A in serum but not in leucocytes. As leucocytes

were a mixed population of lymphocytes and PMN, which have different levels of Hex-B (Ellis and Patrick, 1976), this was not surprising. Although nearly all the hexosaminidase in Sandhoff leucocytes and most of the activity in Sandhoff serum was classified as component A using the DEAE-cellulose batch method, isoelectric focusing studies showed that this was in fact two other components of lower isoelectric point, probably components S and S'. The percentage Hex-A in leucocytes and serum from Sandhoff heterozygotes was above the control range. These findings were compatible with the theories and findings of Beutler, Kuhl et al. (1975) and Beutler and Kuhl (1975), who proposed that Hex-B was a homopolymer of β subunits, whereas Hex-A was a heteropolymer of α and β subunits and that Hex-S was a source of the α subunits. As it was proposed that Sandhoff's disease was attributable to a deficiency of β subunits, heterozygotes would be expected to have half the normal number of β subunits and a normal number of α subunits. A shift to more α,β heteropolymers at the expense of β homopolymers would be predicted. Conversely, Tay-Sachs heterozygotes, with an underproduction of α subunits, would form more homopolymers at the expense of α,β heteropolymers.

# PART 2

# α-D-GLUCOSIDASE

# AND ITS

# DEFICIENCY IN POMPE'S DISEASE

## GLYCOGENOSIS TYPE II (POMPE'S DISEASE) AND ITS ASSOCIATED ENZYME DEFICIENCY.

Glycogen storage diseases were originally categorised into two types which differed clinically. Whereas type I glycogen storage disease had mainly liver involvement (von Gierke, 1929), in type II the heart and muscle were also affected (Pompe, 1932). Type II glycogen storage disease is also known as Pompe's disease or generalised glycogenosis on account of the storage of glycogen in many tissues of the body in this disorder (Hug and Schubert, 1967). It is now recognised that there are a number of other forms of glycogen storage disease (Howell, 1972).

Hers (1963) reported that the liver of patients who had died of Pompe's disease was deficient in acid α-glucosidase activity. Pompe's disease differed from other known glycogen storage diseases in that the stored glycogen was enclosed by a limiting membrane (Baudhuin et al., 1964). Pompe's disease was in fact the first lysosomal storage disease to be recognised. Glycogen accumulates within the lysosome owing to the deficiency of the lysosomal enzyme α-1,4-glucosidase. Whilst glycogen is the substrate of this enzyme in vivo, less complex glycosides may be used as substrates in vitro. The substrate usually used in this thesis was 4-methylumbelliferyl-α-D-glucopyranoside which was shown (Salafsky and Nadler, 1973b) to be appropriate for the diagnosis of Pompe's disease. The enzyme will be termed maltase when the substrate used is maltose and α-glucosidase when fluorogenic or chromogenic substrates have been used.

In classical Pompe's disease, patients usually die of cardiac

failure within one year of birth (Ehlers and Engle, 1963). A milder form of Pompe's disease has been reported in which the principle clinical finding was muscular weakness, patients sometimes living to adulthood (Engel, 1970). The enzymatic relationship of adult and infantile forms of Pompe's disease is uncertain. All the cases studied here were of the infantile form of the disease.

As with the other lysosomal storage disorders, it is possible to diagnose the disease in utero. Antenatal diagnosis has been attempted using amniotic fluid, uncultured amniotic fluid cells and cultured amniotic fluid cells (Nadler and Messina, 1969; Cox et al., 1970; Nadler et al., 1970). Amniotic fluid was shown to be unreliable (Nadler et al., 1970) since normal levels of maltase activity at pH 4.0 may be present despite a deficiency in the cultured amniotic fluid cells. Uncultured amniotic fluid cells were also found to be unsuitable as very low levels of α-glucosidase activity can be found even for a normal fetus (Butterworth et al., 1973).

The presence of substantial maltase activity in the amniotic fluid surrounding an affected fetus prompted investigations into the nature and source of this residual activity (Salafsky and Nadler, 1971). It was already known that Pompe kidney had residual maltase activity (Mekanik et al., 1966; Steinitz and Rutenberg, 1967). This differed from normal liver maltase with respect to its pH optimum and response to turanose, an inhibitor of acid maltase activity. Salafsky and Nadler (1971) reported that

kidney maltase had an optimum of pH 6.0, was stable at 45°C for fifteen minutes and was less affected by turanose than the liver enzyme. The maltase of amniotic fluid also had an optimum of pH 6.0 and was inhibited by turanose to a greater extent than that of kidney, but much less than that of liver. Amniotic fluid maltase differed markedly from the kidney enzyme, however, in its heat stability and was rapidly inactivated at 45°C. On the first two counts, it was possible that the maltase found in kidney was the same as that in amniotic fluid. The heat lability of the amniotic fluid enzyme, however, suggested that this was not the case. It was possible, however, that some component of the amniotic fluid rendered the maltase more labile. It was not stated whether the conditions for heat treatment had been controlled. Indeed, it has been shown (Koster et al., 1976) that liver acid maltase has markedly different heat stability curves when incubated at pH 4.0 and pH 7.4. A study of α-glucosidase in Pompe urine revealed that the pH-activity plot of the enzyme was similar to that of amniotic fluid and kidney (Salafsky and Nadler, 1973a). Urine α-glucosidase from normal adults had a biphasic pH curve with optima at pH 4.0 and pH 5.5 with much the greater activity at pH 4.0. Normal infant urine had a broad α-glucosidase optimum of pH 4.5-5.0 which indicated the presence of more than one enzyme component.

Although kidney is not usually the tissue of choice in studying lysosomal enzyme activities, it was possible that the enzyme found in Pompe kidney might also occur in other tissues particularly leucocytes and amniotic fluid. This enzyme was studied with

special emphasis on its behaviour in the presence of turanose. As maltose and 4-methylumbelliferyl-α-D-glucopyranoside could both be used as substrates, the behaviour of α-glucosidase when the substrates were used in competition was also investigated. The results of these studies will be presented in the thesis.

With the findings of Nadler et al. (1970) and Salafsky and Nadler (1971) that amniotic fluid was unsuitable for the antenatal diagnosis of Pompe's disease, cultured amniotic fluid cells have become the preferred assay material. This technique cannot produce such rapid results as time is required to culture sufficient cells. To overcome this problem, a microtechnique has been developed (Galjaard et al., 1973). Using this technique, it was possible to assay for α-glucosidase activity on as few as seventy cells grown on plastic foil and to obtain a result in 7-14 days. However, difficulties were encountered in that two erroneous diagnoses were made. It was suggested that these difficulties had arisen from the use of primary cell cultures for the test and cells of a later passage as controls. Fujimoto et al. (1976) claimed that the problem was due to the neutral α-glucosidase having significant activity at pH 4.0. They tackled the problem by heat-treating samples to remove the more labile neutral enzyme component. It was doubtful from their results whether any real problem of interference existed. Their procedure also produced inconsistent results in that variable amounts of activity at pH 4.0 were lost and the ratio of activity at pH 4.0 to that at pH 6.0 after heat treatment was not constant.

In leucocytes of Pompe patients, acid maltase has been shown to be deficient (Huijinget al., 1963; Williams, 1966; Nitowsky and Grunfeld, 1967), although sometimes difficulties have been encountered in demonstrating this (Koster et al., 1972). They reported that leucocytes from cases of the milder variant of Pompe's disease were not deficient in acid maltase activity and later this was also shown to be the case in classical Pompe's disease (Koster et al., 1974). It would seem that the problem is due to the method of isolating the leucocytes as the method of earlier workers preferentially isolated lymphocytes, whereas the dextran sedimentation technique employed by Koster preferentially isolated polymorphonuclear cells. Indeed, Koster et al. (1974) showed this to be the cause of these differences. In view of this, it was surprising that Seiler et al. (1973) had reported that for two cases of adult Pompe's disease, the difference between the enzyme activities in cases and controls was more pronounced when total leucocytes were used than when lymphocytes alone were assayed.

Koster et al. (1974) investigated the different components of maltase in polymorphonuclear leucocytes and lymphocytes to try to explain the difficulties encountered in the diagnosis of Pompe's disease. It was shown that when polymorphonuclear cells were used, the maltase activity at pH 4.0 of patients with Pompe's disease overlapped with the levels of obligate heterozygotes and when the ratio of the activity at pH 4.0 to that at pH 6.0 was used, heterozygotes overlapped with both patients and controls. With lymphocytes, there was no overlap of Pompe values at pH 4.0

with the others, but it was not possible to distinguish heterozygotes from controls. The ratio of activity at pH 4.0 to that at pH 6.0 for lymphocytes gave three groups corresponding to patients, obligate heterozygotes and controls. When clinical findings suggest that a patient is suffering from Pompe's disease, a low level on this test would confirm the diagnosis, as carriers are very unlikely to display clinical symptoms similar to those of Pompe's disease. This of course would be a completely unacceptable approach if it were applied to antenatal diagnosis, as there would be no clinical information regarding the fetus in which heterozygous levels could be expected.

Koster et al. (1976) raised antibody against human liver acid maltase and found that the maltase activity of lymphocytes at pH 4.0 was almost completely inhibited by the antibody, but that of polymorphonuclear cells or dextran isolated leucocytes had 20% activity remaining. If polymorphonuclear cells from patients with Pompe's disease were treated with antibody, no inhibition occurred. It would seem, therefore, that although polymorphonuclear cells from Pompe patients have maltase activity at pH 4.0, the component responsible for the degradation of glycogen in vivo (acid maltase) is deficient. When glycogen was used as substrate, activities in both polymorphonuclear cells and dextran isolated leucocytes of Pompe patients were well separated from those of controls. It was possible that the residual maltase activity at pH 4.0 in polymorphonuclear cells of Pompe patients was related to the residual enzyme in Pompe kidney. Pompe kidney, however, had considerable residual activity

towards glycogen as well as maltose (Koster et al., 1976). The nature of the residual α-glucosidase in kidney, leucocytes and amniotic fluid were investigated in the course of this study.

METHODS.

Enzyme assay.

α-Glucosidase was assayed using 4-methylumbelliferyl-α-D-glucopyranoside (1mM). Substrate (2mM), dissolved in disodium hydrogen phosphate-citric acid buffer (0.2M/0.1M), pH 4.0 (McIlvaine, 1921), containing 0.1%$^{v}$/v Triton X-100 and 0.02%$^{w}$/v sodium azide, was mixed with an equal volume of extract. For pH profiles, one volume of aqueous substrate (4mM) was mixed with one volume of double strength buffer and two volumes of extract. The assay was stopped using 0.1M-sodium carbonate and the fluorescence read as described in the appendix (page 242). When studying the effect of inhibitors, these were added by using a reduced volume of extract and making up the volume with inhibitor solution. The effect of citrate buffer and buffer concentration was studied using double strength buffers and substrate as for pH profiles.

Maltase was assayed as follows:-

| | enzyme | substrate blank | extract blank |
|---|---|---|---|
| Maltose in distilled water | 0.025ml | 0.025ml | - |
| Phosphate-citrate buffer, pH 4.0 (0.07M/0.035M) | 0.075ml | 0.075ml | 0.075ml |
| Tissue homogenate (1%$^{w}$/v) | 0.050ml | - | 0.050ml |
| Distilled water | - | 0.050ml | 0.025ml |

After one hour at 37°C, 0.20ml sodium phosphate buffer (0.1M), pH 7.0, was added and the reaction was stopped by heating at 100°C for 5 minutes. An aliquot (50µl) was taken and the glucose

released, estimated by the glucose oxidase method as described in the appendix (page 253 ).

Protein was estimated (Lowry et al., 1951) as described in the appendix (page 243 ).

Enzyme source and preparation of extract.

(i) Cultured cells.

Amniotic fluid cells and skin fibroblasts were cultivated as described in the appendix (page 244 ).

(ii) Blood samples.

The dextran sedimentation method was usually used for the preparation of leucocytes as described in the appendix (page 247 ). If lymphocytes were required, however, these were prepared using Ficoll-Paque, also described in the appendix (page 248 ).

(iii)Post-mortem tissues.

Tissues, liver and kidney, were stored at -40°C until required.

Homogenates in distilled water were made by sonication using a "Soniprobe" (Dawe's Instruments) for both tissues and cells. Tissue homogenates were made up at 1%$^{w}$/v and subsequently diluted a fifth for the α-glucosidase assay. After sonication, the extract was centrifuged at 900g for 5 minutes.

Isoelectric precipitation.

The neutral α-glucosidase was removed by pretreatment with acetate buffer, pH 5.0, followed by centrifugation at 900g for

5 minutes. The final concentration of acetate buffer used for this precipitation/inactivation step was 10mM for cultured cells, 20mM for leucocytes and 50mM for tissues.

## (a) STUDIES ON HUMAN α-GLUCOSIDASES.

### RESULTS.

#### (i) pH profiles of α-glucosidase in different tissues.

The pH profiles of 4-methylumbelliferyl-α-glucosidase for cultured amniotic fluid cells and cultured skin fibroblasts, liver, dextran isolated leucocytes and lymphocytes, kidney and amniotic fluid are shown in Figure 32 (page 94). The characteristic double peak of activity, present in cultured cells and liver, was not apparent in leucocytes, kidney and amniotic fluid and was probably hidden by the presence of an intermediate enzyme activity. As lymphocytes clearly had an optimum of pH 4.0-4.5, they must have little or no intermediate enzyme. Cells from post-mortem kidney were cultured, but these reverted to fibroblast-like cells in pH profile (Figure 32f, page 94).

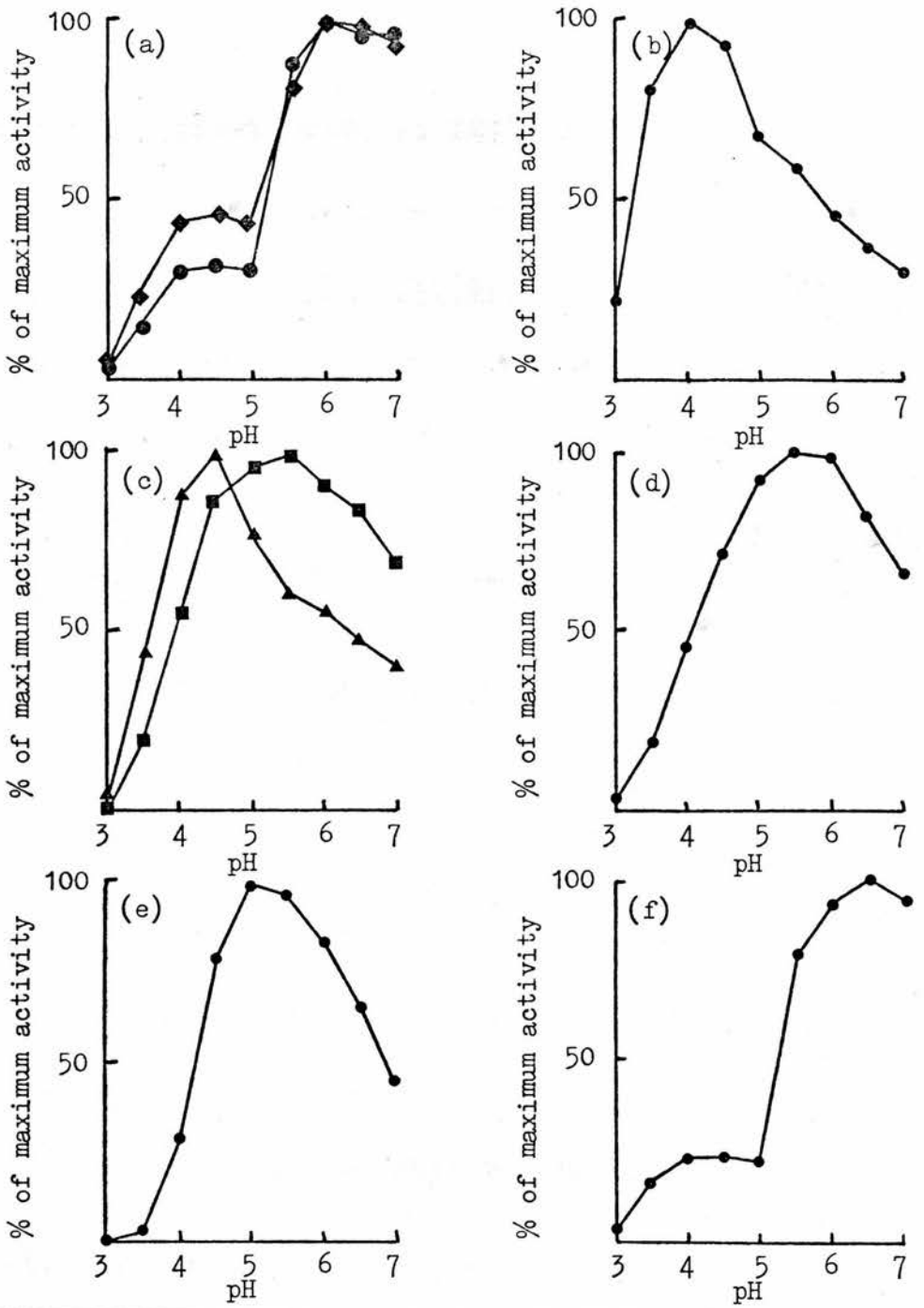


FIGURE 32. α-glucosidase pH profiles of (a) cultured skin fibroblasts (●) and amniotic fluid cells (◆), (b) liver, (c) dextran isolated leucocytes (■) and lymphocytes (▲), (d) kidney, (e) amniotic fluid and (f) cultured kidney cells.

(ii) Precipitation and inactivation of α-glucosidase at pH 5.0.

The neutral form of 4-methylumbelliferyl-α-glucosidase in cultured cells can be precipitated in the presence of sodium acetate buffer (10mM), pH 5.0. The maximum ratio of activity at pH 4.0 to that at pH 6.0 was attained when preincubation at pH 5.0 was used (Table 5 ). Under these optimal conditions for the removal of the neutral α-glucosidase, the loss of activity at pH 4.0 was 8%. When the precipitate was resuspended in sodium phosphate buffer (10mM), pH 7.0, not all the activity was recovered. Precipitation was extremely fast, however, and if the precipitate was resuspended in buffer quickly, little activity was lost. Hence some of the activity can be irreversibly inactivated and not just precipitated.

TABLE 5 Effect of pH treatment on loss of α-glucosidase activity of cultured skin fibroblasts.

| Treatment* | % Enzyme activity | | Specific activity ratio pH 4.0/6.0 |
|---|---|---|---|
| | pH 4.0 | pH 6.0 | |
| 3.5 | 109 | 29 | 3.2 |
| 4.0 | 100 | 24 | 3.5 |
| 4.5 | 89 | 16 | 4.7 |
| 5.0 | 92 | 14 | 5.5 |
| 5.5 | 108 | 29 | 3.2 |
| $H_2O$ unspun | 100 | 100 | 0.9 |

* Cells extracted into water and adjusted to the indicated pH using one volume 20mM-sodium acetate buffer prior to centrifugation.

Precipitation of neutral α-glucosidase in liver and kidney required a buffer of higher ionic strength, as 10mM buffer was not strong enough to maintain the pH of tissue homogenates at pH 5.0. Accordingly, homogenates with a final acetate concentration of 50mM were prepared. For liver, it was found that some of the acid α-glucosidase was precipitated when the higher ionic strength buffer was used (Figure 33a, page 97 ). The removal of the neutral α-glucosidase, however, was quite effective. The effect on kidney homogenates was somewhat different (Figure 33b, page 97 ). Rather more activity was precipitated in the assay range pH 4.0-5.0 than for liver. The activity, not spun down, had an optimum at pH 4.5, but there was a shoulder of activity between pH 5.0 and pH 7.0.

For leucocytes (dextran isolated), a final concentration of 20mM-sodium acetate in the homogenate was used. Precipitation shifted the optimum to a lower pH and removed a large percentage of the α-glucosidase activity (Figure 34 , page 97 ). In the example, the activity at pH 4.0 was reduced to only one fifth of the untreated level, whereas the activity at pH 6.0 was reduced seventeen times.

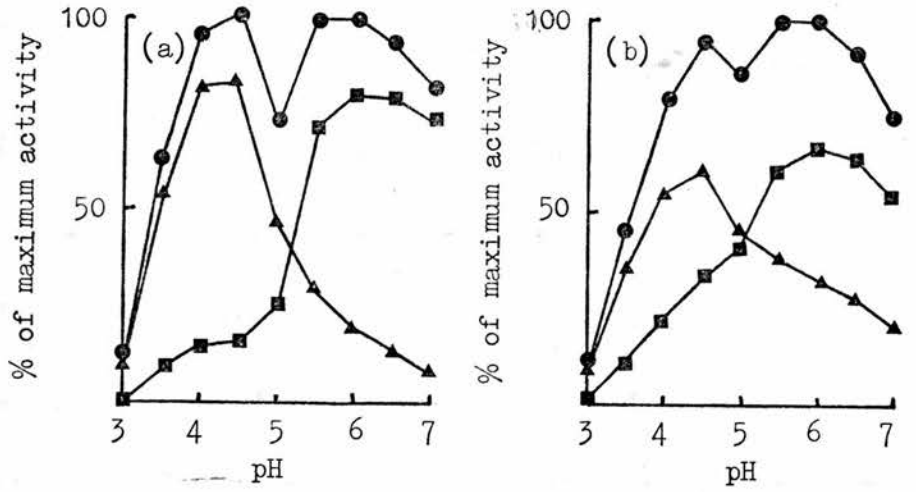


FIGURE 33 . The effect of pretreatment in 50mM-sodium acetate buffer, pH 5.0, and centrifugation at 900g for 5 minutes on the α-glucosidase pH profile of (a) liver and (b) kidney.

(●) total

(■) precipitate, resuspended in 10mM-sodium phosphate, pH 7.0

(▲) difference (calculated supernatant)

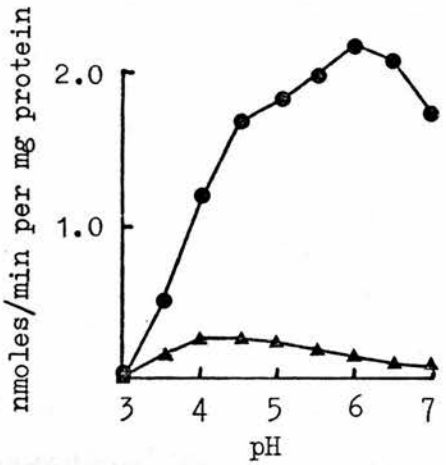


FIGURE 34 . The effect of precipitation using 20mM-sodium acetate buffer, pH 5.0, on the pH profile of leucocyte α-glucosidase.

(●) total

(▲) supernatant at pH 5.0

(iii)Inhibitors of α-glucosidase activity.

The effects of several sugars on the α-glucosidase activity of kidney were tested. The effects of maltose, turanose, α-methyl-glucose and sucrose at a concentration of 30mM, on the pH profile of kidney α-glucosidase are shown in Figure 35 (page 99 ). Sucrose and α-methyl-glucose had an insignificant effect. As expected, turanose was a potent inhibitor of kidney α-glucosidase. Maltose, which is also a substrate for α-glucosidase, was also found to be an inhibitor when 4-methylumbelliferyl-α-D-glucopyranoside was used as substrate. Although the maximum inhibition by turanose was at pH 5.0. it inhibited at all pH values. The peak of inhibition caused by maltose was sharper and was also optimal at pH 5.0, but there was a shoulder of inhibition at pH 4.0. Some activation of the neutral α-glucosidase was noted with α-methyl-glucose and sucrose, but it was not possible to detect whether this was the case with turanose and maltose owing to the massive inhibition of other components in kidney.

The effect of maltose as an inhibitor was investigated in cultured skin fibroblast and liver homogenates at pH 4.0 to determine the effect of the inhibition on the genuine acid α-glucosidase. In Figure 36 (page 100 ), it can be seen that the inhibition was almost identical in fibroblast and liver with 30mM maltose inhibiting 60-65% of acid α-glucosidase activity.

The effect 30mM turanose on homogenates of dextran-isolated leucocytes and cultured skin fibroblasts is shown in Figure 37 (page 100 ). In the presence of inhibitor, the activity in leucocytes had a neutral optimum of pH 6.0-6.5 with a shoulder at

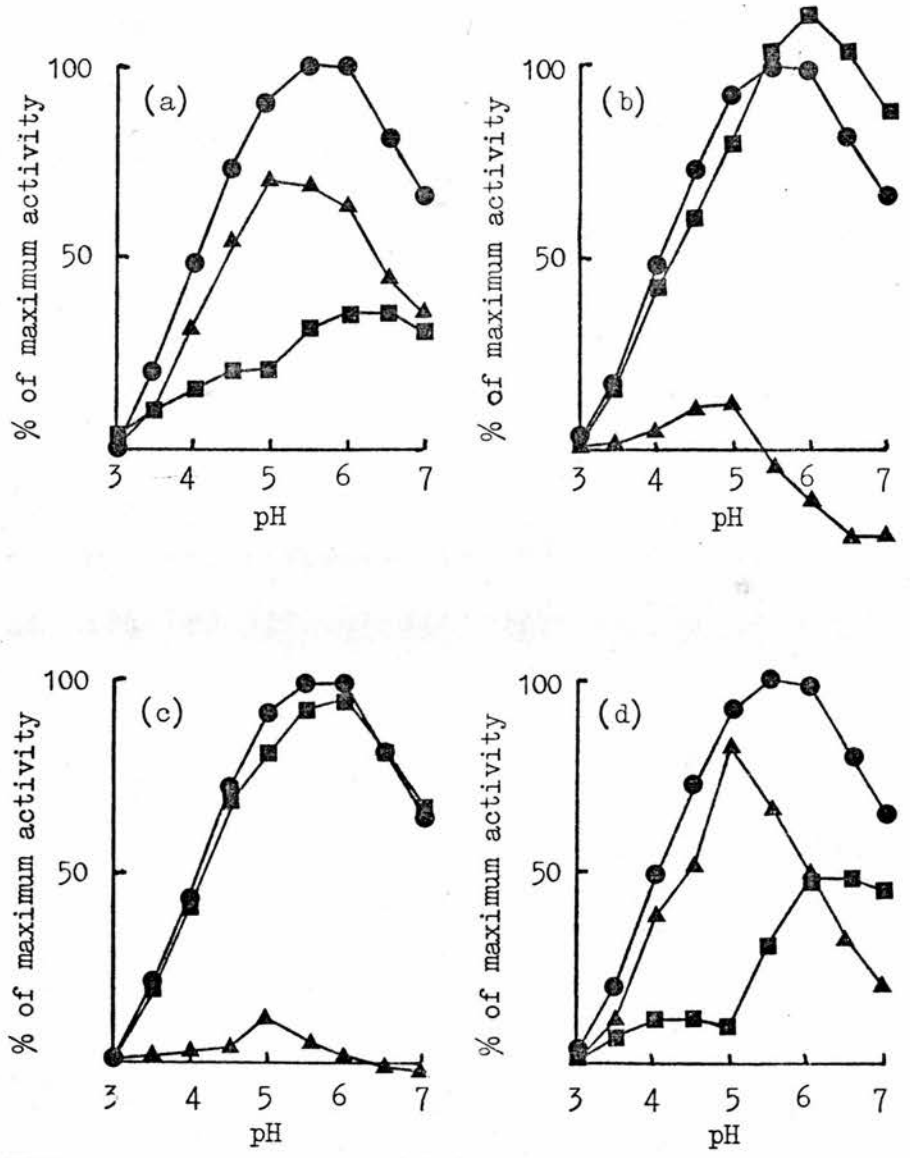


FIGURE 35. Effect of (a) 30mM-turanose, (b) 30mM-α-methyl-glucose, (c) 30mM-sucrose and (d) 30mM-maltose on the α-glucosidase pH profile of control kidney.

(●) no addition

(■) sugar added

(▲) difference plot

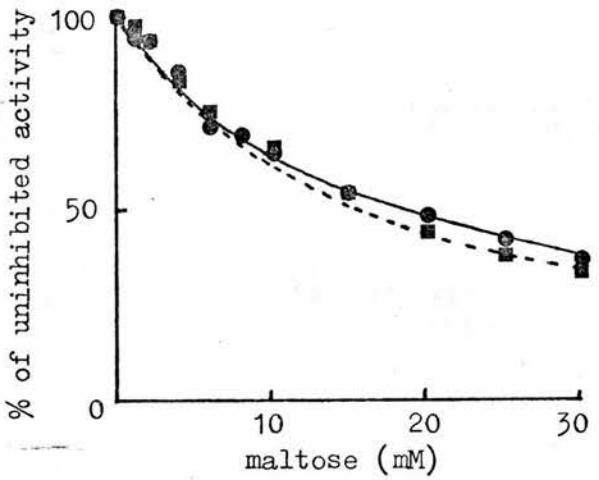


FIGURE 36 . Effect of increasing concentrations of maltose on the acid (pH 4.0) α-glucosidase of fibroblasts (● ———) and liver (■ - - - - -).

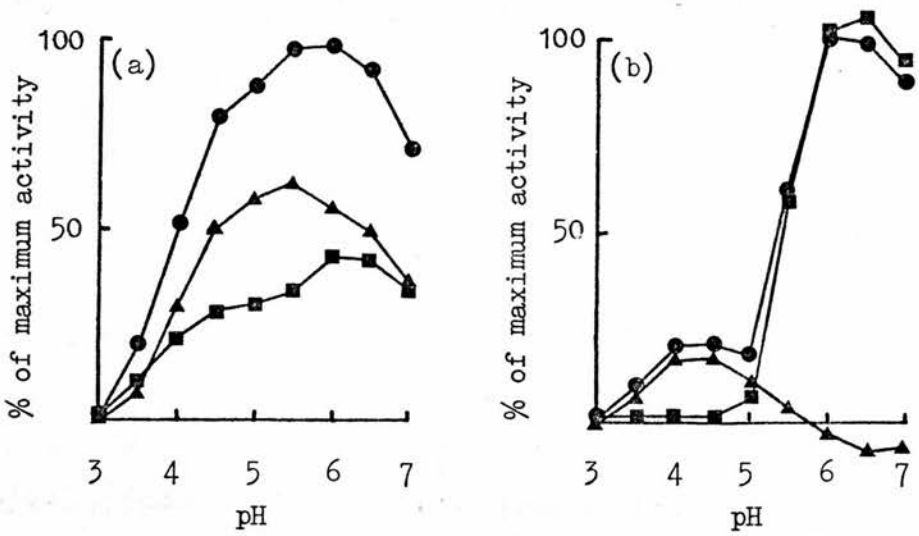


FIGURE 37 . Effect of 30mM-turanose on the α-glucosidase pH profile of (a) dextran isolated leucocytes and (b) cultured skin fibroblasts.

(●) no addition

(■) 30mM-turanose

(▲) difference plot

pH 4.5-5.0. A plot of the difference between the usual profile and the turanose inhibited profile gave a peak of inhibition at pH 5.0-6.0. The effect of 30mM-turanose on cultured skin fibroblast α-glucosidase was more marked than for leucocytes. The acid α-glucosidase was almost totally inhibited and there was some activation of the neutral component.

The effects of 8mM-maltose on the pH profile of kidney and liver homogenates highlighted the difference between these two tissues (Figure 38 ). In liver, the acid α-glucosidase was

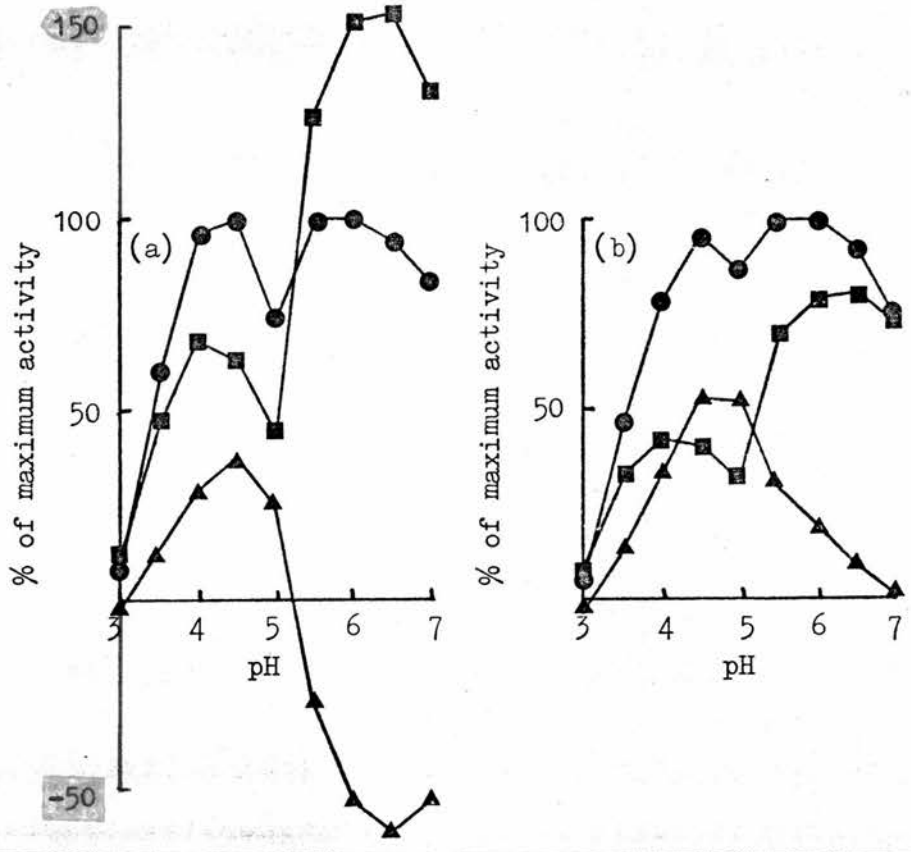


FIGURE 38 . Effect of 8mM-maltose on the α-glucosidase pH profile of (a) control liver and (b) control kidney.

(●) no addition

(■) 8mM-maltose

(▲) difference plot

inhibited by 30%, whereas the activity of the neutral peak was increased by 50-60%. In kidney, the activity at pH 4.0 was inhibited by 40% and the neutral activity by 20%.

(iv) Combination of pH 5.0 precipitation and maltose inhibition.

A summary of the effects of turanose, maltose and pH 5.0 precipitation is shown in Table 6 . The results for maltose in conjunction with acid precipitation for liver homogenates are shown in Figure 39 (page 104 ). The acid component present in the precipitate was partially inhibited by maltose (8mM), whilst the neutral component was markedly activated. Plots (Figure 39 , page 104 ) showing the difference between total activity and precipitated activity, with and without maltose, were calculated. When maltose was not included, all the neutral activity had apparently been removed and only the acid α-glucosidase remained. In the presence of 8mM-maltose, however, the acid component was inhibited, but some activation between pH 6.0 and pH 7.0 occurred. Thus residual neutral α-glucosidase which had not been precipitated could be clearly demonstrated by maltose activation. Not surpris-

TABLE 6 Summary of the effects of turanose, maltose and pH 5.0 precipitation on the components of α-glucosidase.

| treatment | α-glucosidase component | | |
|---|---|---|---|
| | acid | intermediate | neutral |
| turanose | - - - - | - - | + + + |
| maltose | - | - - - | + + + |
| pH 5.0 precipitation | - | - - - | - - - - - |

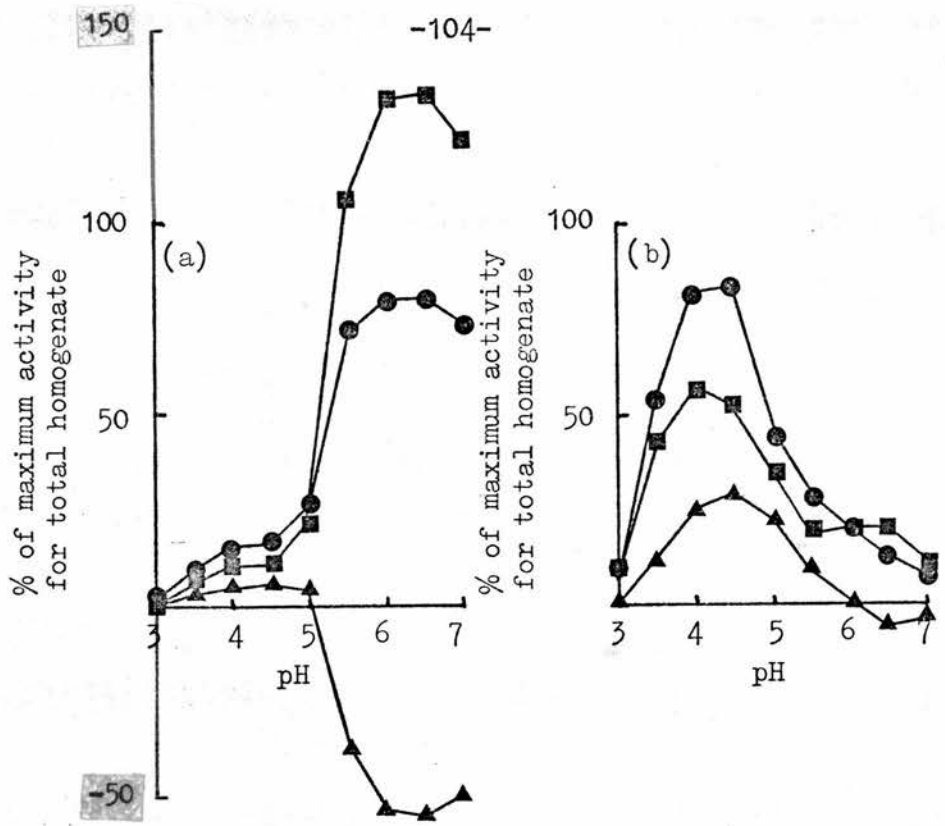


FIGURE 39 . Effect of 8mM-maltose on the α-glucosidase pH profile of liver; (a) the precipitate after pretreatment with 50mM-sodium acetate buffer, pH 5.0, and (b) the calculated supernatant.

(●) no addition

(■) 8mM-maltose

(▲) difference plot

ingly, the results using kidney homogenates were different (Figure 40 , page 105 ). The precipitated material, as for liver, had a pH optimum in the neutral region, but a larger shoulder was found at acid pH. When the precipitate was assayed in the presence of 8mM-maltose, the α-glucosidase activity at pH 4.0 was reduced by about 60%, whereas the activity at pH 6.0 was barely affected. The activation of the neutral component, if present, had been cancelled out by the inhibition of the acid and intermediate components, the activity of which tailed into the neutral range. The difference plot (Figure 40 , page 105 ) between total activity

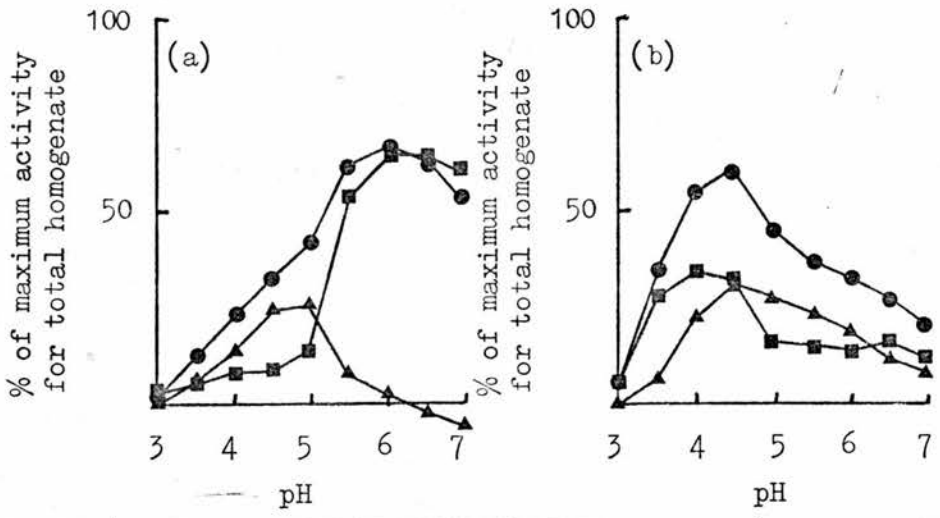


FIGURE 40 . Effect of 8mM-maltose on the α-glucosidase pH profile of kidney; (a) the precipitate after pretreatment with 50mM-sodium acetate buffer, pH 5.0, and (b) the calculated supernatant.

(●) no addition

(■) 8mM-maltose

(▲) difference plot

and precipitated activity (ie. calculated supernatant activity) gave a profile with a peak at pH 4.5 and shoulder at pH 5.5-6.0. Assay in the presence of maltose shifted the optimum for this difference plot to pH 4.0 with a loss of activity of 39% at pH 4.0 and 58% at pH 6.0. Thus a combination of pH 5.0 precipitation and maltose inhibition almost totally removed the intermediate and neutral α-glucosidase activities.

DISCUSSION.

(i) A form of α-glucosidase with pH optimum between the acid and neutral components of the enzyme.

It was clear that cultured skin fibroblasts and liver homogenates had acid and neutral α-glucosidase activities. It had been noted that the kidney of patients with Pompe's disease was apparently not deficient in acid α-glucosidase (Mekanik et al., 1966). Similar results were found for amniotic fluid (Salafsky and Nadler, 1971) and dextran isolated leucocytes (Koster et al., 1974). It was possible that these problems arose because of the presence of a component of α-glucosidase with a pH optimum between those of the acid and neutral α-glucosidases, but with considerable activity at pH 4.0. It was seen quite clearly that the α-glucosidase pH profiles of kidney, leucocytes and amniotic fluid differed from those of cultured cells and liver. Kidney cells grown in tissue culture reverted to fibroblast type, so it was clear that this intermediate form of α-glucosidase could not be maintained in tissue culture.

(ii) Use of pH 5.0 precipitation for fibroblast extracts.

The precipitation of neutral α-glucosidase from extracts of cultured skin fibroblasts was very effective and showed that the contribution of the neutral component in cultured cells to the activity at pH 4.0 was negligible. It was of interest that other workers (Fujimoto et al., 1976) considered that the presence of the neutral enzyme led to problems when undertaking antenatal diagnosis using cultured amniotic fluid cells. Their solution to the problem was the differential heat inactivation of the neutral component. This seemed very unsatisfactory as variable amounts of activity at pH 4.0 were lost and the ratio of activity at pH 4.0 to that at pH 6.0 was very variable. With pH 5.0 precipitation, this ratio was remarkably constant. As the neutral α-glucosidase appeared to have negligible activity at pH 4.0 and was considerably more labile than the acid component, the wisdom of any pretreatment of cultured cell extracts is questionable. When diagnosis was undertaken by pH profile, the presence of a peak of activity at pH 6.0 was a useful internal control and showed that the extract had not been harshly treated.

(iii)Use of pH 5.0 precipitation for kidney and leucocyte extracts.

The use of pH 5.0 precipitation for dextran isolated leucocytes and kidney was more useful than for fibroblasts, but not as effective. The use of buffer of higher ionic strength for tissue homogenates was necessary, but created problems. The pH profile of the supernatant was distorted by the pH 5.0 buffer and so this was not assayed direct, but calculated from the profiles of the total homogenate and the precipitate. It was realised that some of the neutral α-glucosidase might be inactivated permanently by pH 5.0 precipitation, but when all operations were performed quickly, loss of activity seemed to have been kept to a minimum. Dialysis to remove the pH 5.0 buffer was not considered, as the neutral α-glucosidase had been lost on previous occasions when dialysed.

When used for kidney, pH 5.0 precipitation appeared to be partially effective in removing the intermediate α-glucosidase component from solution. An identical experiment with liver showed that the method also precipitated some of the acid α-glucosidase when this ionic strength (50mM) was used and it must be assumed, therefore, that in kidney some of the acid α-glucosidase was probably precipitated. It was difficult to be sure whether all the neutral component had been removed, but as was stressed earlier, this was not important to the activity at pH 4.0. Experiments with maltose, which will be discussed later (page 110), suggested that a small amount of neutral component was not precipitated. The apparent activation by maltose of the enzyme activity at pH 6.0, calculated to be in the supernatant, was attributed to

the presence of the neutral component. The pH 5.0 precipitation method was, therefore, of limited value when working with kidney.

The effectiveness of the removal of most of the α-glucosidase activity in leucocytes with a marked switch to a more acid pH optimum by pH 5.0 precipitation suggested that only a small proportion of the α-glucosidase in dextran isolated leucocytes was the genuine acid component. It was disappointing that the hidden level of acid α-glucosidase was so low, but the usefulness of the precipitation step was exemplified in this instance. These findings were markedly different from those of Koster et al. (1976) who found that the percentage of enzyme precipitated from extracts of dextran isolated leucocytes and polymorphonuclear cells by antibody specifically raised against acid α-glucosidase was around 80%.

(iv) Inhibitors of α-glucosidase activity.

Turanose inhibited liver α-glucosidase more effectively than the activity in kidney at pH 4.0 as was also found by Salafsky and Nadler (1971). Other suitable inhibitors were sought, which might perhaps inhibit the enzyme in kidney more than that in liver. Maltose was found to be suitable for this purpose. This was not surprising, as it was also a suitable substrate for the enzyme. It was necessary to lower the concentration of maltose from the original concentration of 30mM that had been used in order to lessen the extent of the acid α-glucosidase inhibition, which had been demonstrated in control liver. An unusual finding was the stimulation of neutral α-glucosidase activity by both turanose maltose, which had also been noted for α-methyl glucose and sucrose. This phenomenon was difficult to explain, but was possibly due to the stabilisation of this labile enzyme.

It was observed that maltose and pH 5.0 precipitation had similar, but limited effects on the acid α-glucosidase, both suppressed the intermediate component and they had opposite effects on the neutral enzyme. Turanose did not differentiate sufficiently between the acid and intermediate components. As the removal of the intermediate component was the prime concern, the joint effects of pH 5.0 precipitation and maltose inhibition were investigated. It was found that, although some of the acid α-glucosidase was lost, when these techniques were used together the intermediate and neutral components were almost totally removed.

(b) THE α-GLUCOSIDASE DEFICIENCY IN POMPE'S DISEASE.

RESULTS.

(i) Diagnosis of Pompe's disease in liver and cultured cells.

Pompe's disease may be diagnosed using certain tissues simply by the assay of 4-methylumbelliferyl-α-D-glucosidase at pH 4.0. The diagnosis is, however, best illustrated by pH profile. In Figure 41 (page 112 ), α-glucosidase was assayed in the range pH 3.0-7.0 using liver, cultured skin fibroblasts and cultured amniotic fluid cells, showing that cases of Pompe's disease were easily distinguished from controls. Fibroblasts from some obligate heterozygotes for Pompe's disease were not so easily distinguishable from cases and extreme care was necessary when undertaking antenatal diagnosis. Indeed, amniotic fluid cells from a low heterozygote for Pompe's disease were encountered (Figure 41 c, page 112 ) during an antenatal diagnosis and the outcome of the pregnancy was successfully predicted.

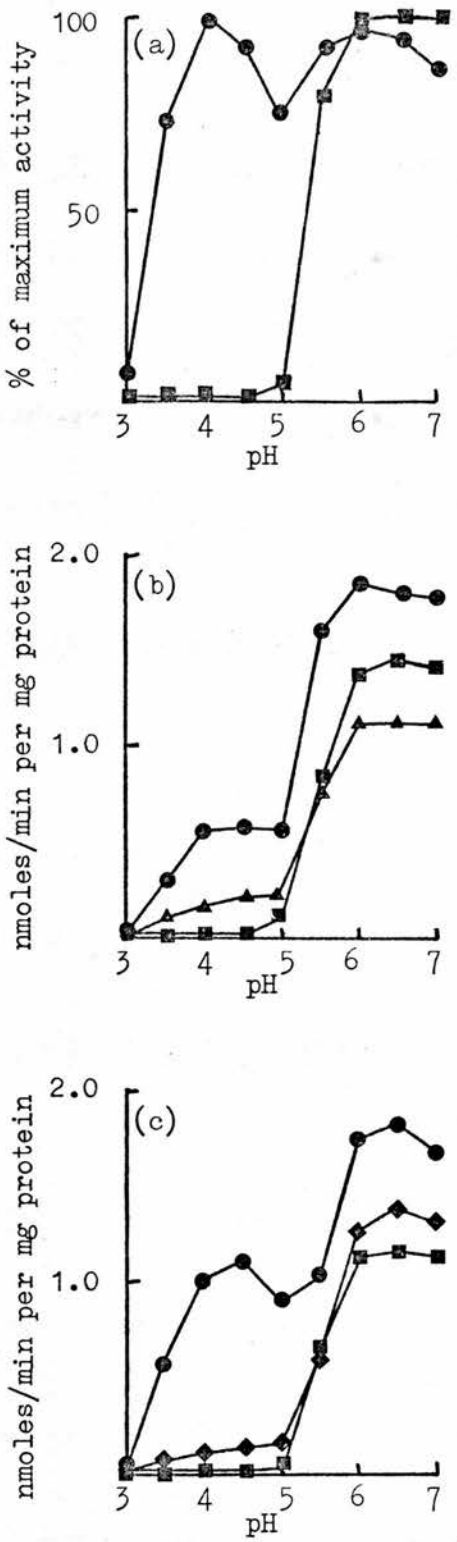


FIGURE 41 . Diagnosis of Pompe's disease by the assay of α-glucosidase using (a) liver, (b) cultured skin fibroblasts and (c) cultured amniotic fluid cells.

(●) control (■) Pompe patient

(▲) obligate heterozygote (◆) presumed heterozygote

(ii) The presence of α-glucosidase activity at pH 4.0 in kidney and leucocytes of patients with Pompe's disease.

Although acid α-glucosidase was profoundly deficient in liver and cultured cells of Pompe patients, there was activity at pH 4.0 in kidney and dextran isolated leucocytes (Figure 42 ).

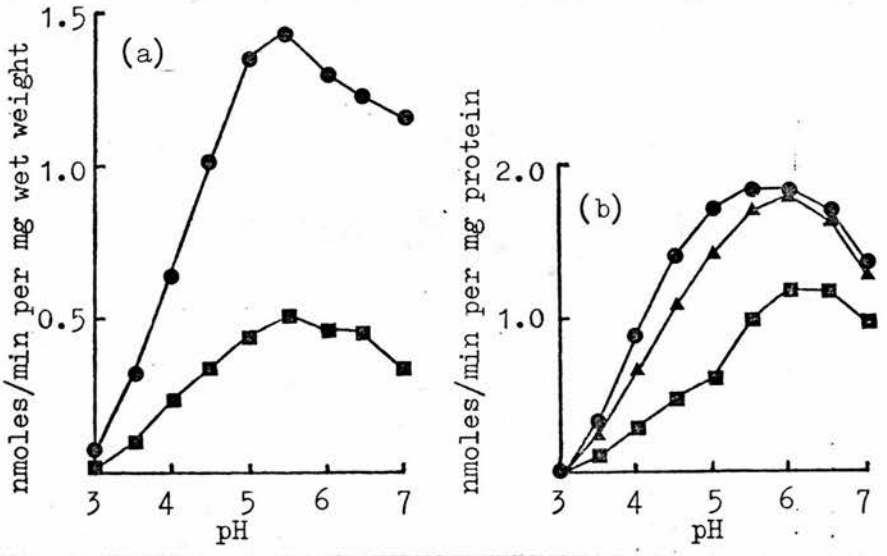


FIGURE 42 . α-glucosidase pH profile of (a) kidney and (b) dextran isolated leucocytes demonstrating the residual activity in Pompe's disease.

(●) control

(■) Pompe's disease

(▲) obligate heterozygote

(iii)Effect of buffer concentration on α-glucosidase activity.

When determining the apparent $K_m$ of control liver acid α-glucosidase and the residual activity of Pompe kidney at pH 4.0, the concentration of the assay buffer was found to be of importance. Although the overall activity of control liver acid α-glucosidase was little affected, the $K_m$ of this enzyme and of the residual enzyme in Pompe kidney was raised with increased buffer concentration (Figure 43 , page 115 ). This was not simply due to increased ionic strength, as assay in acetate buffers of increasing concentration did not give rise to similar changes (Figure 44 , page 116 ). Increasing the concentration of citrate buffer did not alter the $K_m$ of Pompe kidney α-glucosidase, but the $K_m$ of the enzyme in control liver was again raised (Figure 45 , page 117 ). However, the α-glucosidase activity of Pompe kidney was much reduced in citrate, compared with phosphate-citrate buffer.

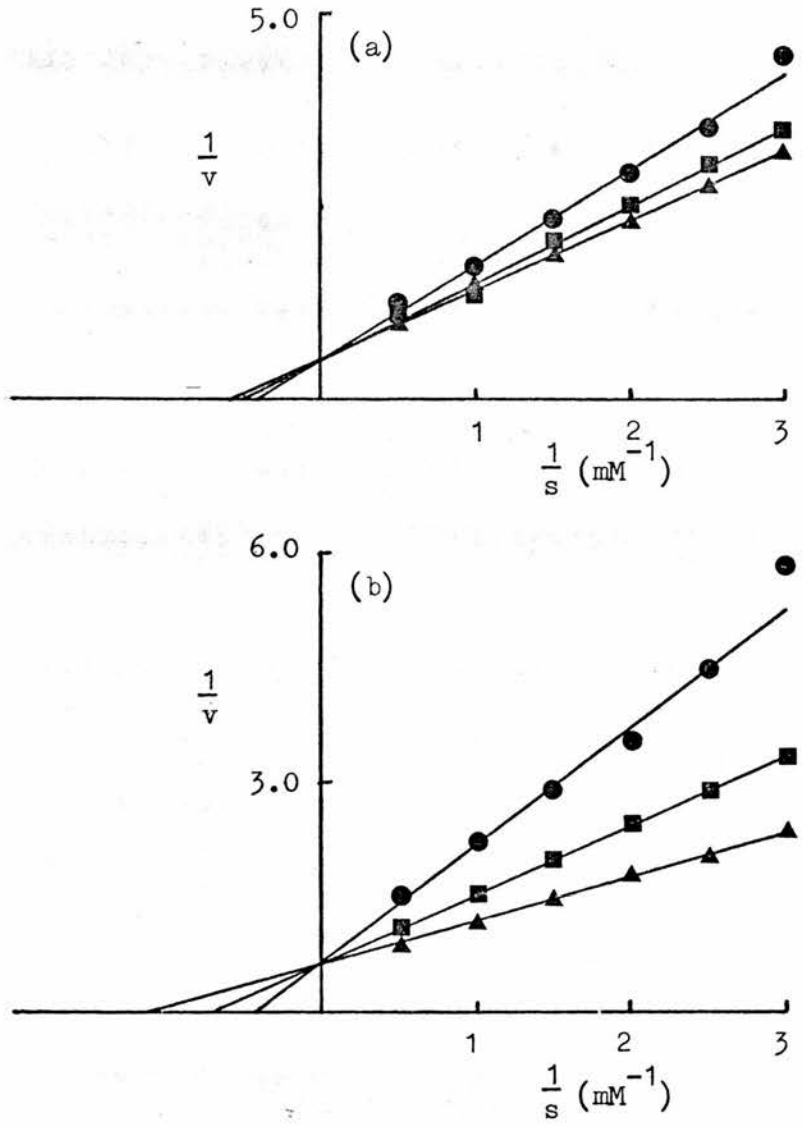


FIGURE 43 . Lineweaver-Burk plots of α-glucosidase at pH 4.0 from (a) control liver and (b) Pompe kidney assayed in phosphate-citrate buffers of final concentration 0.2M/0.1M (●), 0.1M/0.05M (■) and 0.05M/0.025M (▲).

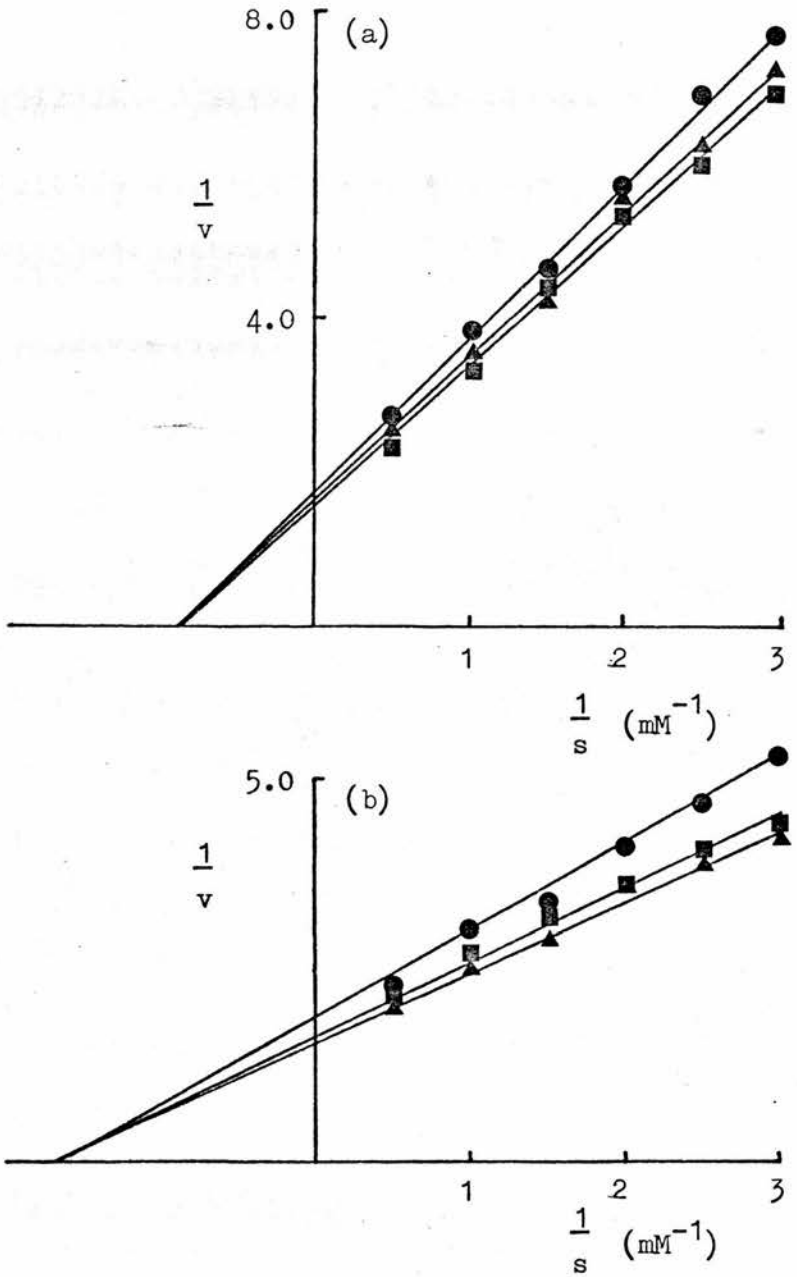


FIGURE 44 . Lineweaver-Burk plots of α-glucosidase at pH 4.0 from (a) control liver and (b) Pompe kidney assayed in acetate buffers of final concentration 0.2M (●), 0.1M (■) and 0.05M (▲).

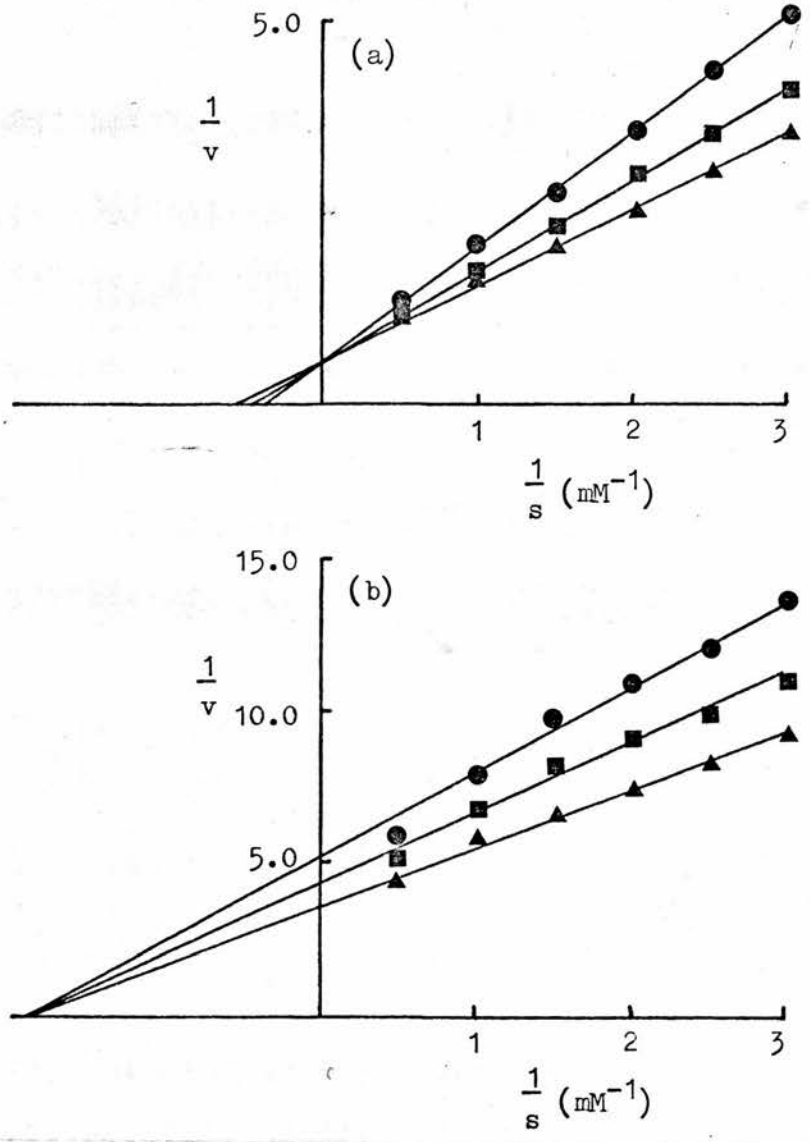


FIGURE 45 . Lineweaver-Burk plots of α-glucosidase at pH 4.0 from (a) control liver and (b) Pompe kidney assayed in citrate buffers of final concentration 0.2M (●), 0.1M (■) and 0.05M (▲).

(iv) Kinetics of turanose inhibition for control liver and Pompe kidney α-glucosidase at pH 4.0.

Plots of $\frac{1}{v}/i$ for different substrate concentrations and $\frac{1}{v}/\frac{1}{s}$ for different turanose concentrations, showed that inhibition was competitive using both phosphate-citrate (Figure 46 ) and

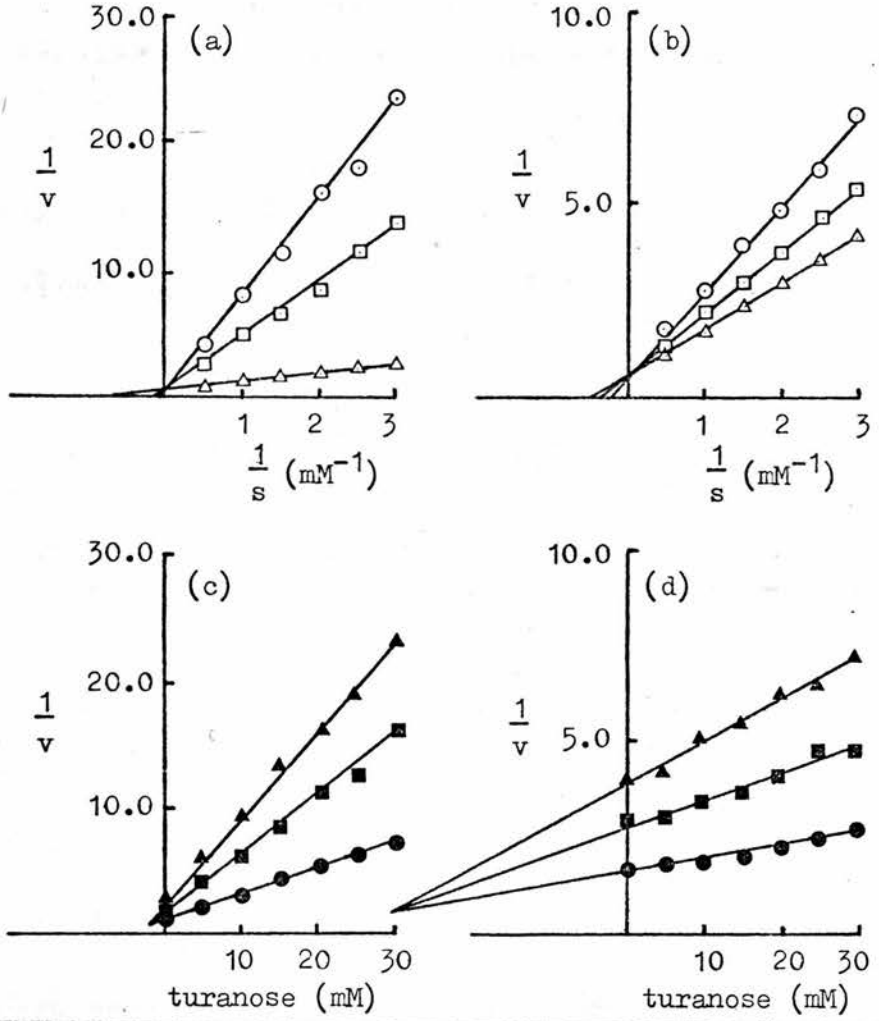


FIGURE 46 . Lineweaver-Burk plots of α-glucosidase in phosphate-citrate buffer (0.2M/0.1M final), pH 4.0, (a and b) for different turanose concentrations 0 (△), 15mM (□) and 30mM (○), and Dixon plots (c and d) for different substrate concentrations 0.33mM (▲), 0.5mM (■) and 1mM (●) using control liver (a and c) and Pompe kidney (b and d).

citrate buffers (Figure 47 ) for either control liver or Pompe kidney.

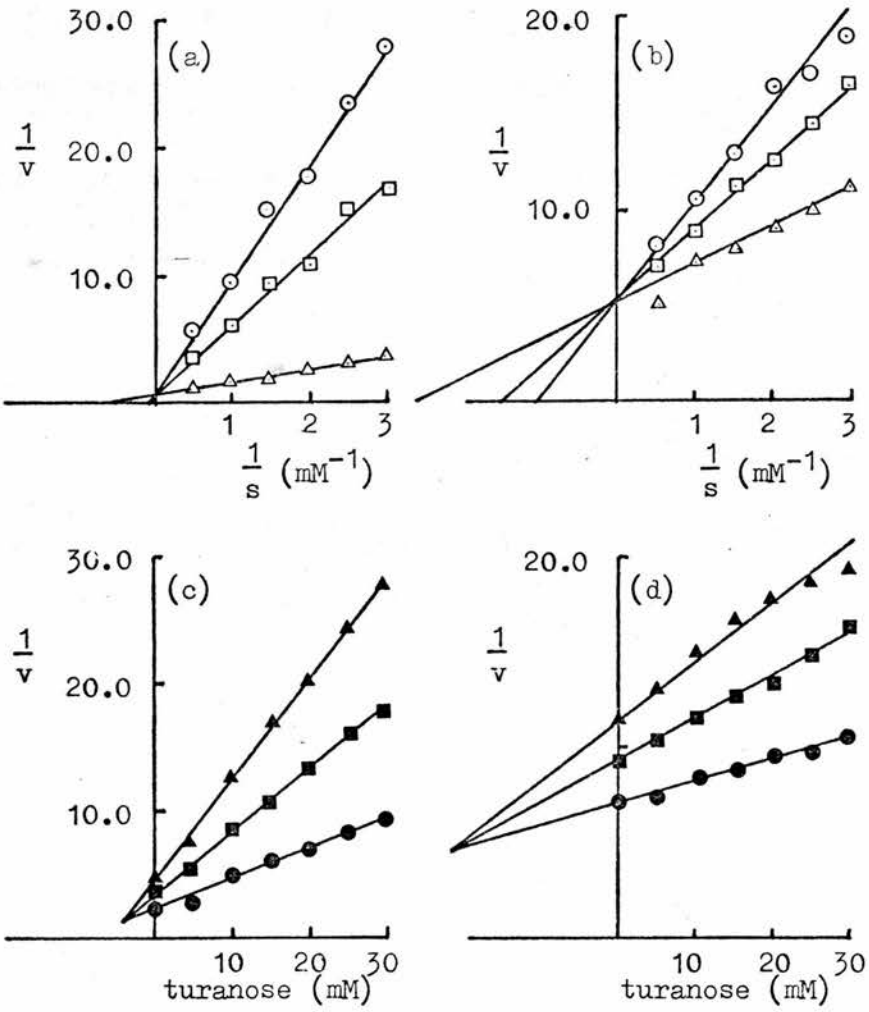


FIGURE 47 . Lineweaver-Burk plots of α-glucosidase in citrate buffer (0.2M final), pH 4.0, (a and b) for different turanose concentrations 0 (△), 15mM (□) and 30mM (○), and Dixon plots (c and d) for different substrate concentrations 0.33mM (▲), 0.5mM (■) and 1mM (●) using control liver (a and c) and Pompe kidney (b and d).

(v) Kinetics of 4-methylumbelliferyl-α-D-glucosidase at pH 4.0 in the presence of maltose.

When assayed in the presence of maltose up to a concentration of 30mM in phosphate-citrate buffer, 4-methylumbelliferyl-α-D-glucosidase was inhibited competitively when the enzyme source was control liver and non-competitively when Pompe kidney enzyme was assayed (Figure 48 ). When citrate buffer was used the

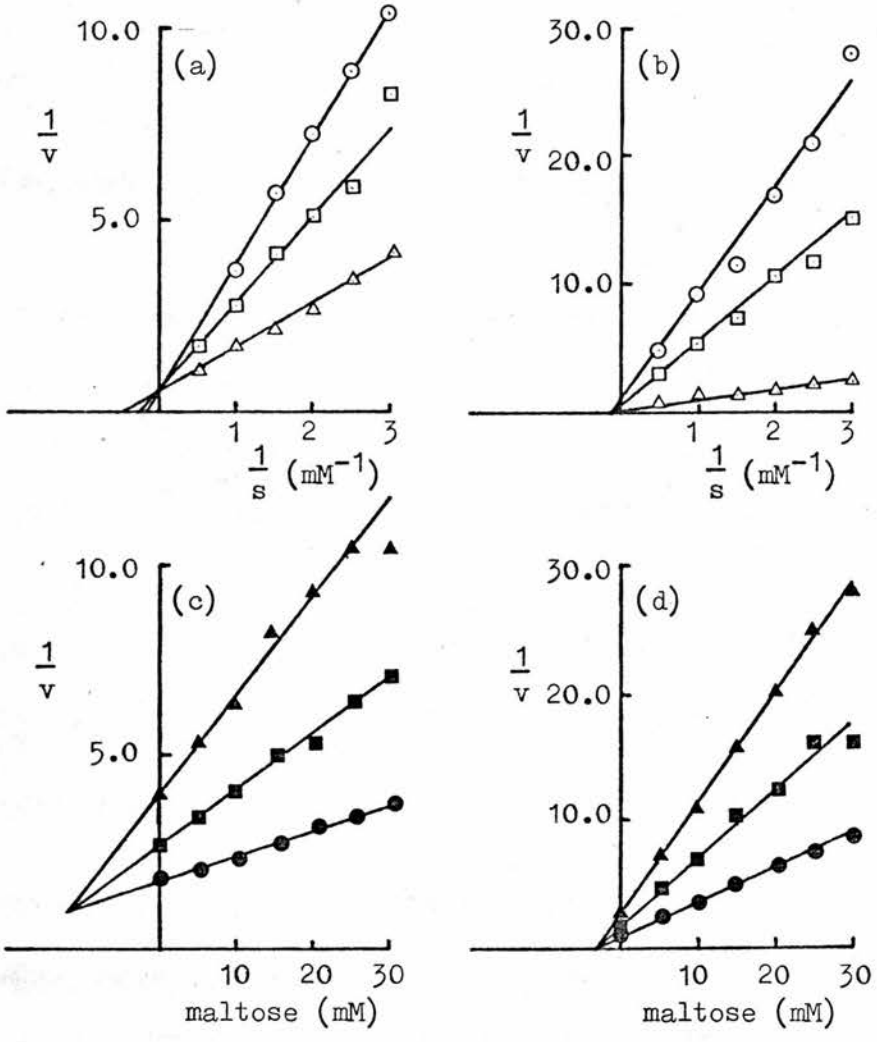


FIGURE 48 . Lineweaver-Burk plots of α-glucosidase in phosphate-citrate buffer (0.2M/0.1M final), pH 4.0, (a and b) for different maltose concentrations 0 (△), 15mM (□) and 30mM (○), and Dixon plots (c and d) for different substrate concentrations 0.33mM (▲), 0.5mM (■) and 1mM (●) using control liver (a and c) and Pompe kidney (b and d).

inhibition was competitive for both control liver and Pompe kidney (Figure 49 ).

When the $K_m$ for control liver and Pompe kidney maltase at pH 4.0 was determined (Figure 50 , page 122 ), it was found that the enzyme in Pompe kidney had much the lower $K_m$.

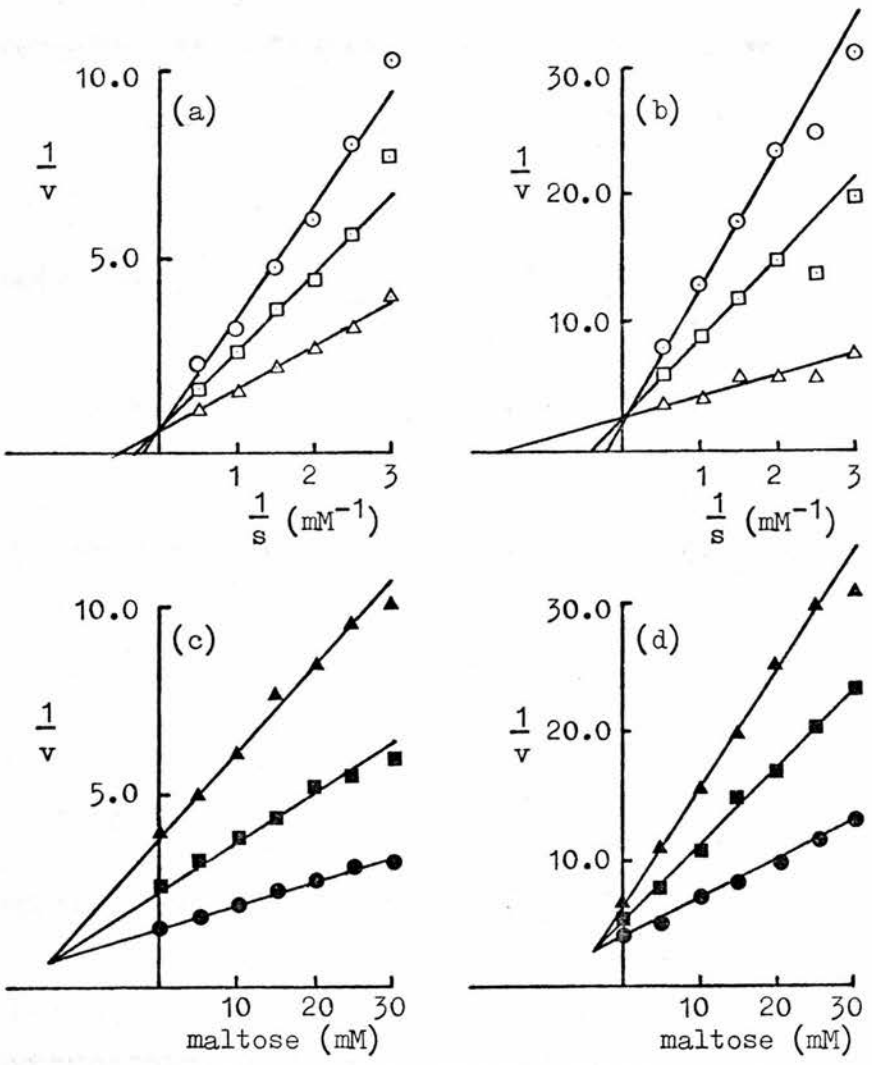


FIGURE 49 . Lineweaver-Burk plots of α-glucosidase in citrate buffer (0.2M final), pH 4.0, (a and b) for different maltose concentrations 0 (△), 15mM (□) and 30mM (○), and Dixon plots (c and d) for different substrate concentrations 0.33mM (▲), 0.5mM (■) and 1mM (●) using control liver (a and c) and Pompe kidney (b and d).

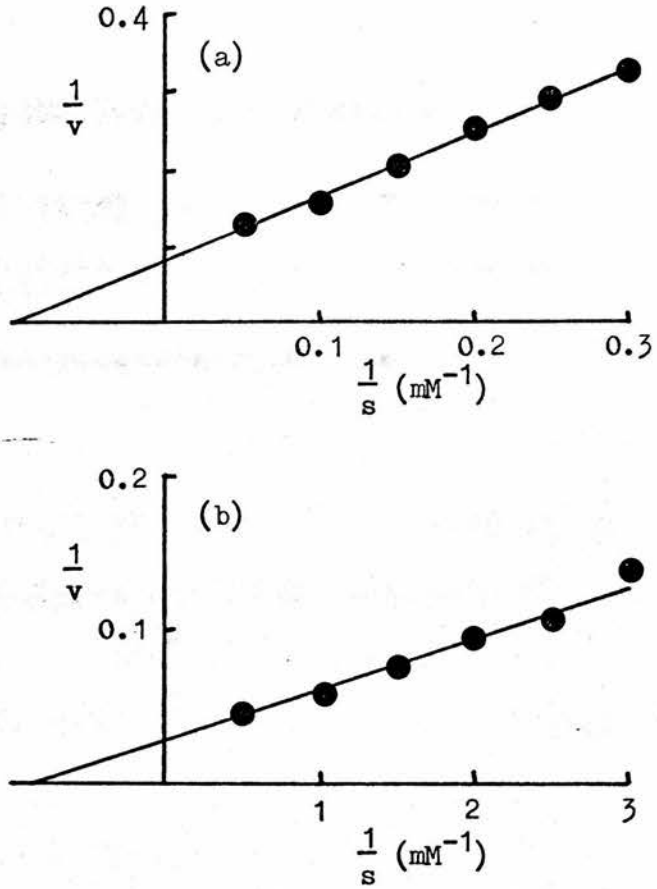


FIGURE 50. Lineweaver-Burk plots of maltase activity at pH 4.0 for (a) control liver ($K_m$ = 10.16) and (b) Pompe kidney ($K_m$ = 1.19).

(vi) Influence of phosphate on the inhibition of Pompe kidney α-glucosidase by citrate.

The inhibition of Pompe kidney α-glucosidase at pH 4.0 by citrate (0.2M), compared with phosphate-citrate (0.2M/0.1M), was 74% using 1mM substrate and, whereas phosphate-citrate buffer was a competitive inhibitor, citrate buffer was a non-competitive inhibitor. The influence of phosphate, adjusted to pH 4.0, on citrate inhibition of an extract dialysed overnight against 10mM-sodium citrate, pH 6.0, was investigated. A plot of α-glucosidase activity against phosphate concentration for different citrate concentrations showed that only a low phosphate concentration was required to lift the inhibition (Figure 51 ) and that, apart from

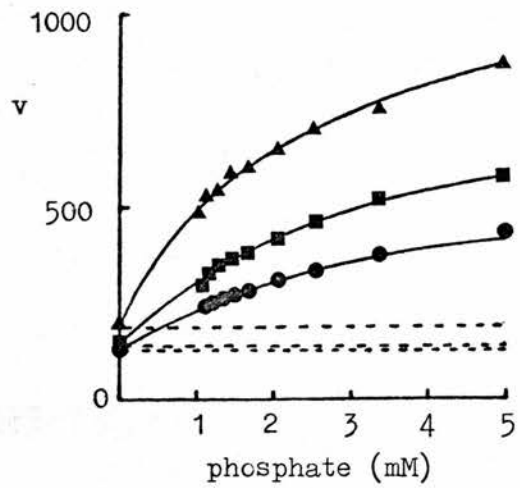


FIGURE 51. Reversal of citrate inhibition of α-glucosidase by phosphate for different citrate concentrations 0.05M (▲), 0.125M (■) and 0.2M (●). Activity in the absence of phosphate at each citrate concentration $v_o$ (------).

not starting at zero activity, the plot was similar to that of a plot of enzyme activity against substrate concentration. When the activity at zero phosphate concentration ($v_o$) was subtracted from activities with phosphate, a plot similar to that for $\frac{v}{s}$ was obtained. A double reciprocal plot suggested that phosphate and citrate were competing for the same site (Figure 52 ).

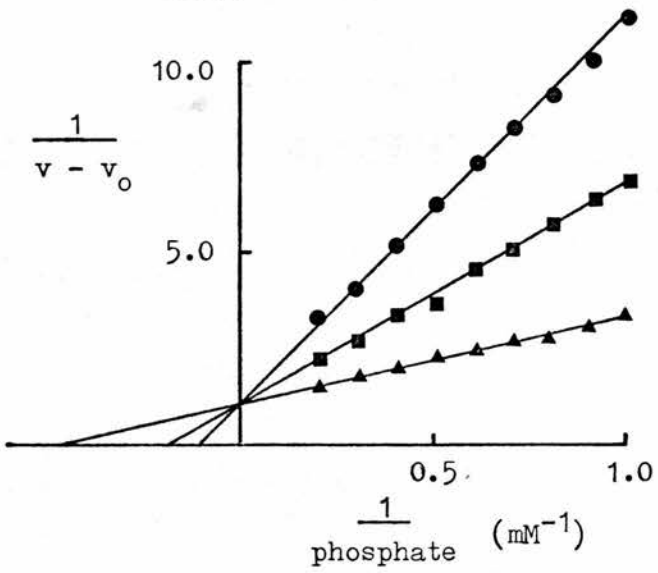


FIGURE 52 . Double reciprocal plot showing competitive reversal of citrate inhibition of Pompe kidney α-glucosidase by phosphate at different citrate concentrations 0.05M (▲), 0.125M (■) and 0.2M (●).

(vii) Application of the inhibitory effects of turanose, maltose and citrate to the detection of Pompe's disease in kidney and the extension of these methods to leucocytes.

The inhibitory effects of 30mM-turanose, 4mM-maltose and 0.2M-citrate on 4-methylumbelliferyl-α-D-glucosidase activity at pH 4.0 were quite different for control liver and Pompe kidney (Table 7 ). Whereas control liver α-glucosidase was affected most severely by turanose, the enzyme in Pompe kidney was especially susceptible to inhibition by maltose and citrate.

A model system was produced by mixing known proportions of liver and Pompe kidney homogenates to simulate the situation in control kidney. The percentage inhibition for each inhibitor was plotted against each of the others (Figure 53 , page 126 ).

TABLE 7 Inhibition (%) of α-glucosidase activity at pH 4.0 of control liver and Pompe kidney.

| Tissue | Turanose (30mM) | Maltose (4mM) | Citrate (0.2M) |
|---|---|---|---|
| Control liver | 87 | 18 | 21 |
| Pompe kidney | 49 | 63 | 81 |

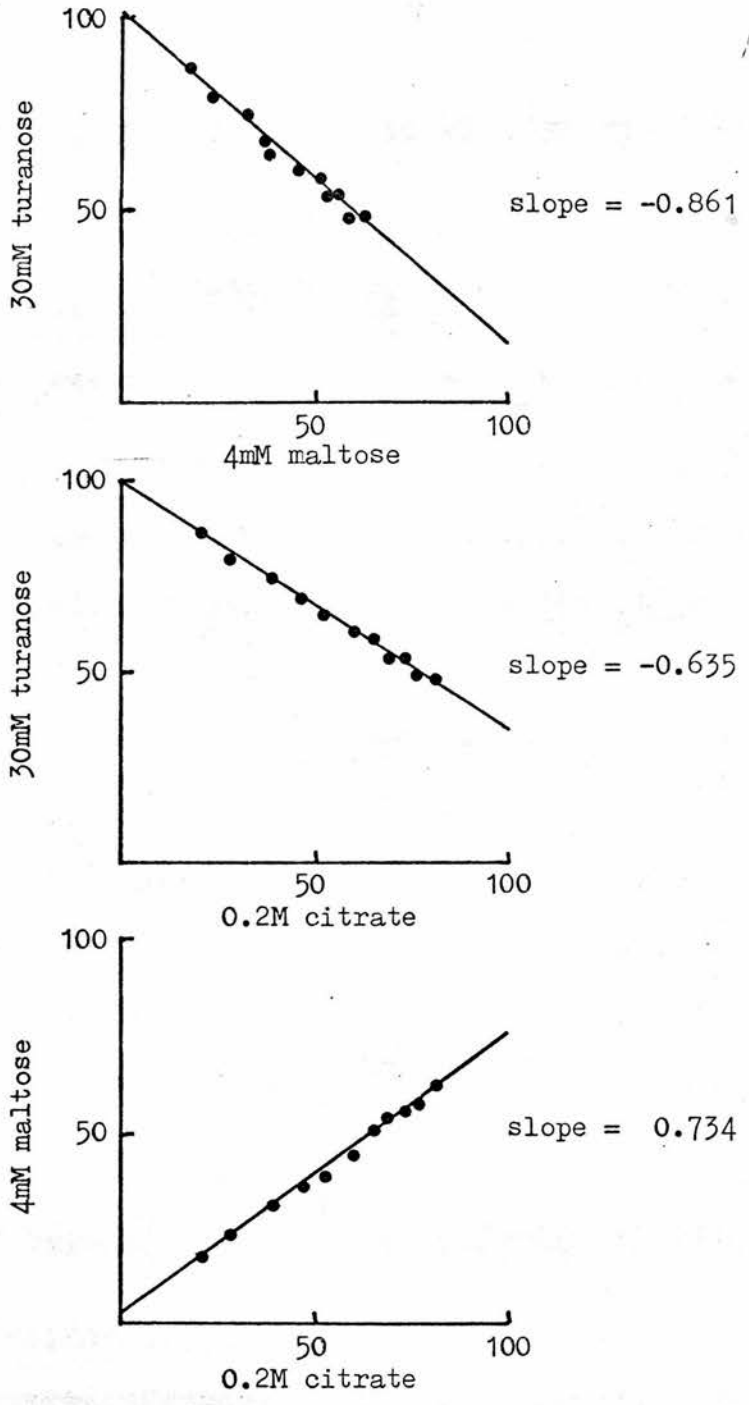


FIGURE 53. The relationship of the % inhibition of α-glucosidase by turanose (30mM), maltose (4mM) and citrate (0.2M) in a series of mixtures of Pompe kidney with control liver.

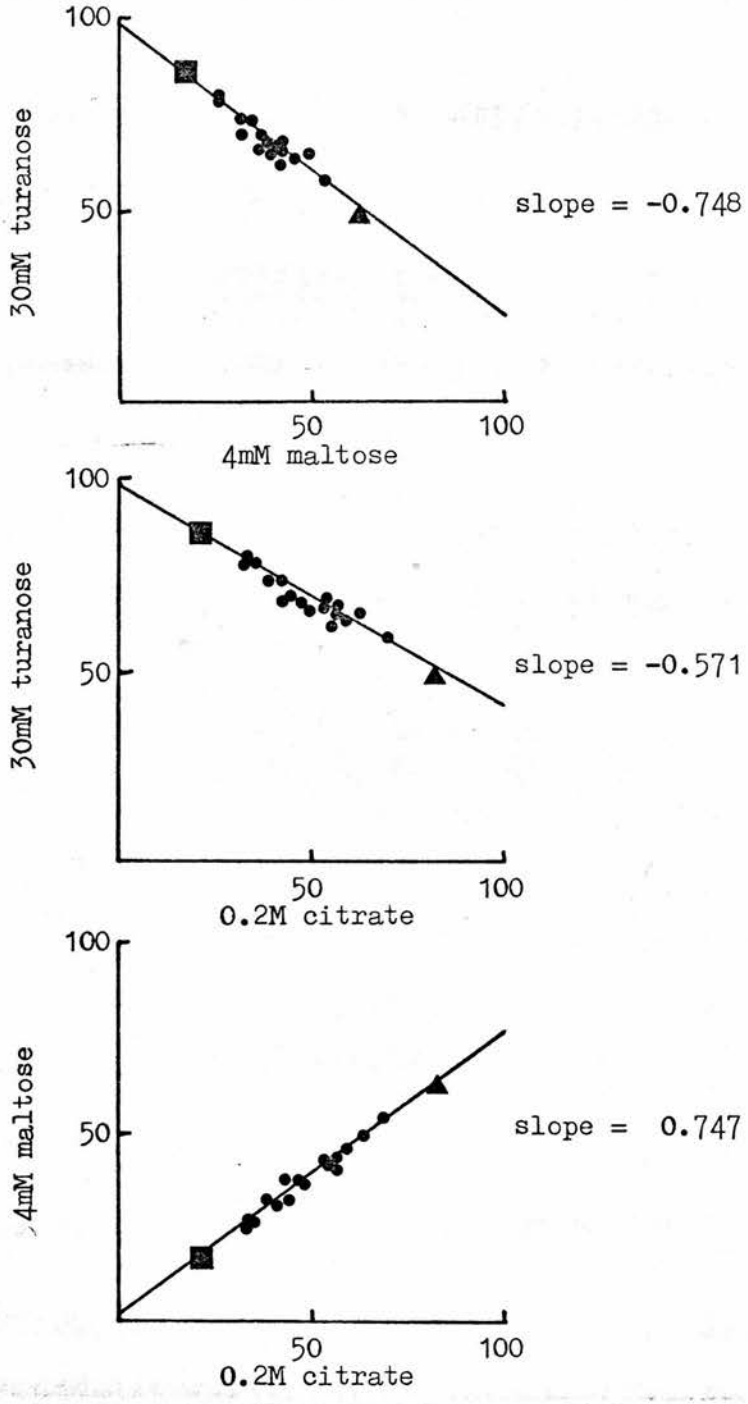


FIGURE 54 . The relationship of the % inhibition of α-glucosidase by turanose (30mM), maltose (4mM) and citrate (0.2M) in homogenates from eighteen control kidneys (•), a Pompe kidney (▲) and control liver (■).

The percentage inhibition by turanose was inversely proportional to that by maltose and citrate, whereas the effects of maltose and citrate were directly related. Eighteen control kidney homogenates were assayed in the presence of these inhibitors and the results (Figure 54 , page 127 ) were in good agreement with those of the model system.

When the known percentage of liver acid α-glucosidase in the model system was plotted against percentage inhibition, straight line plots were obtained for each of the three inhibitors (Figure 55 ). Having calculated the best straight line by regression, it was possible to estimate the percentage of the acid α-glucosidase component present in each kidney homogenate by three different methods (Table 8 , page 129 ). The range of α-glucosi-

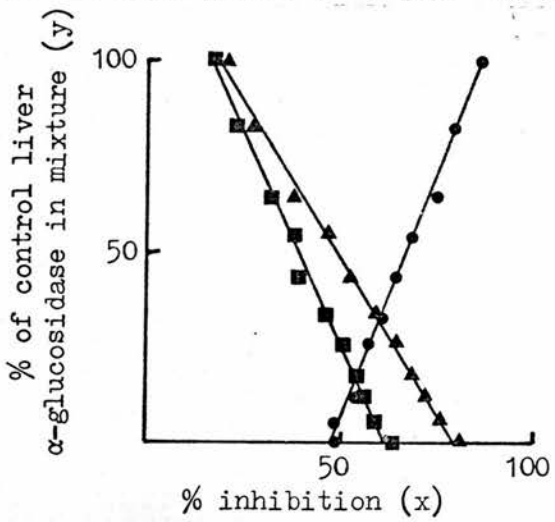


FIGURE 55. The relationship of the % inhibition of α-glucosidase by the three inhibitors to the activity of acid α-glucosidase in the Pompe kidney-control liver mixtures.

(•) Turanose (30mM) $y = 2.56x - 123$

(■) Maltose (4mM) $y = -2.23x + 137$

(▲) Citrate (0.2M) $y = -1.63x + 131$

TABLE 8 Acid α-glucosidase of kidney: Percentage of total α-glucosidase, estimated using the equations for the three inhibitors (Figure 55 , page 128 ).

| Sample | Turanose (30mM) | Maltose (4mM) | Citrate (0.2M) |
|---|---|---|---|
| 1 | 43 | 48 | 40 |
| 2 | 49 | 43 | 45 |
| 3 | 51 | 43 | 45 |
| 4 | 82 | 79 | 77 |
| 5 | 77 | 79 | 74 |
| 6 | 46 | 43 | 40 |
| 7 | 56 | 66 | 59 |
| 8 | 43 | 43 | 41 |
| 9 | 46 | 57 | 51 |
| 10 | 66 | 68 | 64 |
| 11 | 38 | 43 | 41 |
| 12 | 54 | 54 | 61 |
| 13 | 28 | 17 | 19 |
| 14 | 41 | 34 | 35 |
| 15 | 79 | 81 | 77 |
| 16 | 66 | 63 | 67 |
| 17 | 43 | 28 | 28 |
| 18 | 51 | 54 | 54 |

dase activities (nmoles/min per mg protein) for control kidney (n=18) was 1.87-13.45 with a mean (±SD) of 5.22 (±2.47); the activity in Pompe kidney (4.60) was well within the control range. The range of acid α-glucosidase activities of these kidneys estimated from the results in Table 8 (page 129 ) was 1.29-3.73 with mean (±SD) of 2.51 (±0.61). The estimated acid α-glucosidase activity of the Pompe kidney was obviously low (0.05) as it had been used as zero standard in the derivation of the equations, but it should be possible to detect the deficiency in kidney from other cases of Pompe's disease.

Homogenates of eighteen dextran isolated leucocyte preparations were also assayed for α-glucosidase activity at pH 4.0 in the presence of the three inhibitors and the results were similar to those for kidney (Figure 56 , page 131 ). This suggested that the α-glucosidase components in leucocytes were the same as those in kidney and that the equations derived from the model system could also be applied to leucocytes (Table 9 , page 132 ). The range of activities (nmoles/min per mg protein) for control leucocytes (n=18) was 0.44-2.67 with a mean (±SD) of 1.44 (±0.60) and that the acid component estimated from Table 9 (page 132 ) was 0.19-0.92 with mean (±SD) of 0.43 (±0.20). The estimation of acid α-glucosidase activity would enable the diagnosis of Pompe's disease in these cells.

Using this method it was possible to show that lymphocytes were suitable for the diagnosis of Pompe's disease (Figure 56 , page 131 ; Table 10 , page 133 ). It was clear from preliminary studies (Table 11 , page 133 ) that the equation was not applic-

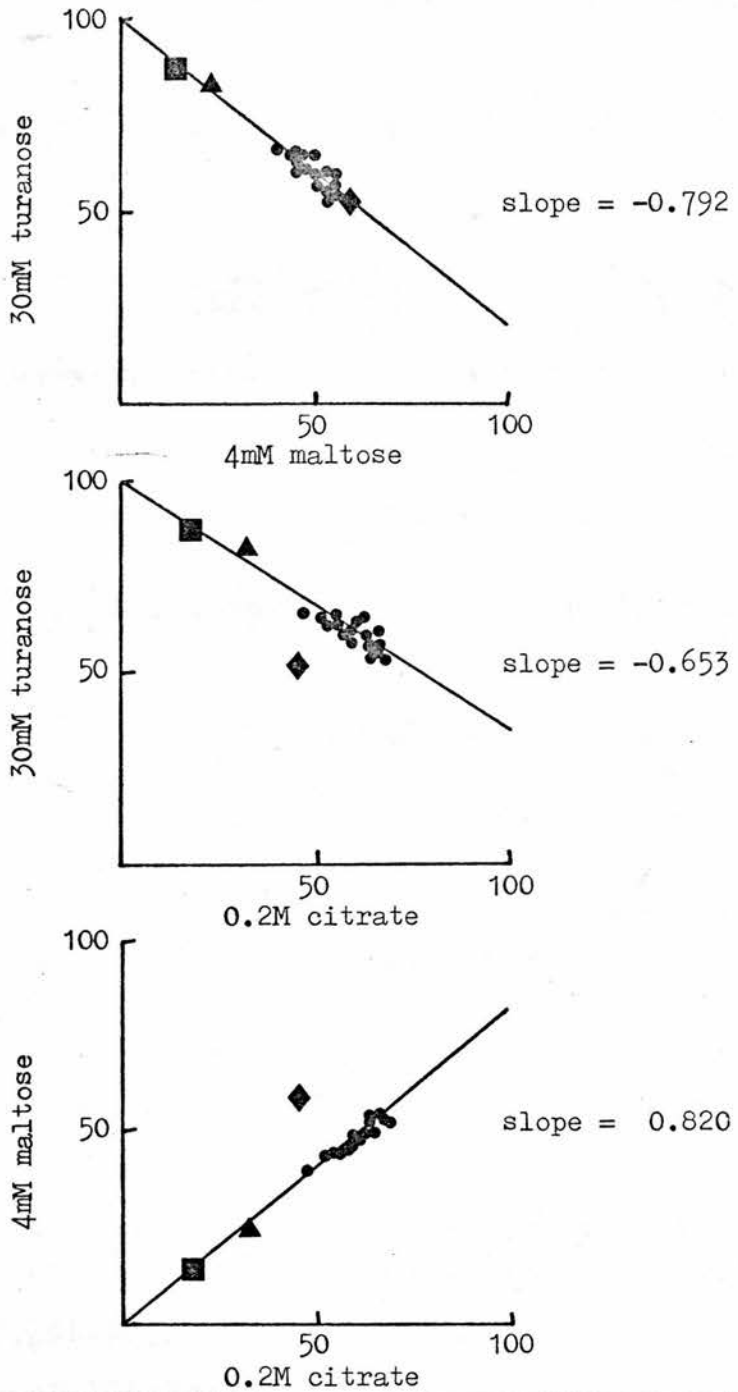


FIGURE 56. The relationship of the % inhibition of α-glucosidase by turanose (30mM), maltose (4mM) and citrate (0.2M) in homogenates of white blood cells.

(■) Lymphocytes; Dextran isolated leucocytes, (•) total homogenates, (▲) supernatant after pH 5.0 precipitation, (♦) precipitate after pH 5.0 precipitation.

able to amniotic fluid.

TABLE 9 Acid α-glucosidase of leucocytes: Percentage of total α-glucosidase activity, estimated using the equations for the three inhibitors (Figure 55 , page 128 ).

| Sample | Turanose (30mM) | Maltose (4mM) | Citrate (0.2M) |
|---|---|---|---|
| 1 | 23 | 27 | 27 |
| 2 | 31 | 14 | 25 |
| 3 | 18 | 19 | 25 |
| 4 | 41 | 30 | 32 |
| 5 | 33 | 32 | 35 |
| 6 | 31 | 34 | 35 |
| 7 | 43 | 37 | 41 |
| 8 | 23 | 17 | 23 |
| 9 | 13 | 19 | 27 |
| 10 | 39 | 38 | 42 |
| 11 | 41 | 39 | 48 |
| 12 | 31 | 17 | 28 |
| 13 | 25 | 28 | 35 |
| 14 | 43 | 26 | 30 |
| 15 | 46 | 49 | 55 |
| 16 | 15 | 15 | 23 |
| 17 | 35 | 37 | 45 |
| 18 | 34 | 31 | 37 |

TABLE 10 Purity (%) of acid α-glucosidase in the preparation estimated using the inhibitors.

| Sample | Turanose (30mM) | Maltose (4mM) | Citrate (0.2M) |
|---|---|---|---|
| A) Leucocytes | | | |
| 1) pH 5.0 Supernatant | 90 | 85 | 78 |
| 2) pH 5.0 Precipitate | 10 | 7 | 57 |
| B) Lymphocytes | 100 | 107 | 102 |

TABLE 11 Inhibition (%) of amniotic fluid α-glucosidase at pH 4.0 by maltose, turanose and citrate.

| Sample | Turanose (30mM) | Maltose (4mM) | Citrate (0.2M) |
|---|---|---|---|
| undialysed | 24 | 5 | 68 |
| dialysed | 33 | 9 | 77 |

(viii) pH 5.0 precipitation as an aid in the diagnosis of Pompe's disease using dextran isolated leucocytes.

As described previously (page 97 ), the activity of α-glucosidase in dextran isolated leucocytes was massively reduced particularly at pH 6.0 after precipitation/inactivation at pH 5.0. In a known case of Pompe's disease, difficulties were encountered in confirming the diagnosis by assay of α-glucosidase in leucocytes. After the precipitation step, the activity of the sample under investigation was clearly separated from that of controls (7% of mean) as was that of the proband's mother (38% of mean) (Figure 57 ).

When the enzyme activity of the pH 5.0 supernatant of control dextran isolated leucocytes was tested with the three inhibitors, its behaviour was consistent with a high degree of purity of the acid α-glucosidase (Table 10 , page 133 ; Figure 56 , page 131 ).

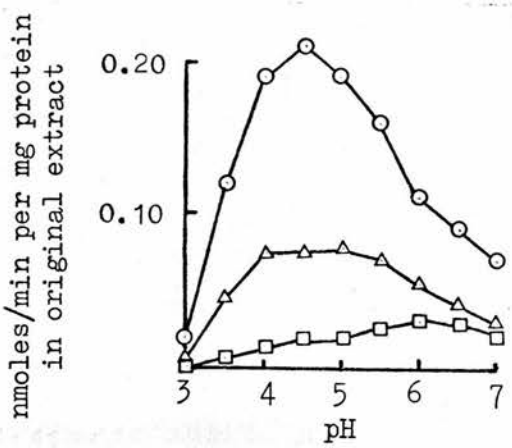


FIGURE 57 . α-glucosidase pH profiles of the supernatant after pH 5.0 precipitation of leucocytes from a typical control (○), a case of Pompe's disease (□) and a Pompe heterozygote (△).

Also shown are the results of inhibition studies on the pH 5.0 precipitate, resuspended in 10mM-sodium phosphate buffer, pH 7.0. Although the percentage inhibition using turanose was consistent with that using maltose, the inhibition using citrate was less than that predicted from the results using the other two inhibitors. The results using turanose and maltose were consistent with a very low level of actual acid α-glucosidase in the precipitate.

DISCUSSION.

(i) Diagnosis of Pompe's disease in liver and cultured cells and difficulties encountered using kidney and leucocytes.

Using liver or cultured cells it was easy to differentiate between cases of Pompe's disease and controls by the assay of 4-methylumbelliferyl-α-D-glucosidase activity. When antenatal diagnosis using cultured amniotic fluid cells is being undertaken, the importance of first studying the heterozygous levels of enzyme activity in the family concerned has been clearly demonstrated. It was not found to be necessary to inactivate the neutral α-glucosidase activity either by heat treatment (Fujimoto et al., 1976) or by precipitation at pH 5.0 as described in this thesis (page 95 ). Difficulties in the antenatal diagnosis of Pompe's disease experienced by Niermeijer et al. (1975) were probably attributable to a lack of sufficient information on the lower limits of heterozygous α-glucosidase activity.

Kidney and dextran isolated leucocytes were unsuitable if diagnosis was attempted simply by the assay of α-glucosidase at pH 4.0 and pH profiles were also of little use in clarifying the situation. This had been shown previously for acid maltase in kidney (Mekanik et al., 1966) and in dextran isolated leucocytes (Koster et al., 1974). These latter workers found that lymphocytes were suitable for diagnosis by the assay of acid maltase.

(ii) The effects of inhibitors on control liver and Pompe kidney α-glucosidase at pH 4.0.

The effect of phosphate-citrate buffer concentration on α-glucosidase activity, particularly that of Pompe kidney, was complex. This was not simply due to the ionic strength of the buffer, as changes in acetate buffer concentration had little effect. It was not possible to test the effect of phosphate buffer, as pH 4.0 was outside its buffering range. Although increasing citrate concentration did not alter the $K_m$ of Pompe kidney α-glucosidase, citrate was a potent inhibitor of this enzyme. The slight competitive inhibition of control liver acid α-glucosidase by phosphate-citrate buffer was attributable to the citrate component. As citrate alone was a more potent inhibitor of Pompe kidney α-glucosidase than when phosphate was present, it would seem that phosphate can lift citrate inhibition. The reversal of inhibition appeared to be due to competition between phosphate and citrate for the site at which citrate inhibited α-glucosidase activity non-competitively. The very low levels of phosphate required were indicative that phosphate had much more affinity for this site than citrate. At the concentrations of phosphate-citrate used for the assay of the enzyme, this site would be saturated with phosphate, thus nullifying the non-competitive component of citrate inhibition. Increasing the phosphate-citrate concentration would then be equivalent to increasing the effective citrate concentration, with no component of inhibition at the non-competitive site. Inhibition would, therefore, be restricted to the hydrolytic site and be competitive.

Turanose was a competitive inhibitor of control liver and Pompe kidney α-glucosidase at pH 4.0 in either phosphate-citrate or citrate buffer. When maltose was used as an inhibitor of 4-methylumbelliferyl-α-D-glucosidase activity, the control liver enzyme was inhibited competitively as found by De Barsy et al. (1972), but refuted by Koster and Slee (1977). For Pompe kidney, when the assay buffer was changed from phosphate-citrate to citrate, inhibition was changed from non-competitive to competitive. As the Pompe kidney enzyme had more affinity for maltose than the control liver enzyme, it was surprising that inhibition of the former was of the non-competitive type (phosphate-citrate buffers). It has been suggested (de Burlet and Sudaka, 1977) that the actual function of this kidney enzyme is to transfer one glucose unit from one maltose molecule to another, producing one glucose molecule and one molecule of maltotriose. Two sites would probably be necessary for this transglucosylation reaction and it is likely that the non-competitive inhibition of Pompe kidney α-glucosidase is due to the binding of maltose to the non-hydrolytic site of this enzyme. It would seem that phosphate, having excluded citrate from the non-competitive inhibition site, allows maltose to occupy this site, causing non-competitive inhibition. In the absence of phosphate, citrate would prevent non-competitive inhibition by maltose and consequently, the competition between maltose and 4-methylumbelliferyl-α-D-glucopyranoside for the hydrolytic site would become apparent.

(iii) Use of inhibitors in the diagnosis of Pompe's disease in kidney and leucocytes.

As the inhibitory effect of maltose was complicated when α-glucosidase was assayed in citrate buffer, only phosphate-citrate buffer was used for further inhibition studies with maltose and turanose. The assay of α-glucosidase in citrate buffer was included as an extra parameter. Control liver α-glucosidase was introduced into homogenates of Pompe kidney to enable study of the inhibitory effects of turanose, maltose and citrate for known mixtures of control liver and Pompe kidney α-glucosidases. It was shown that this model represented the situation in control kidney homogenates and also applied to homogenates of dextran isolated leucocytes. Using the plots obtained from the model system, it was possible to estimate the activity of acid α-glucosidase in kidney and leucocytes, but not in amniotic fluid. The finding, that the residual α-glucosidase found in kidney, but not in liver and cultured cells from Pompe patients, was similar to that in leucocytes in its response to the three inhibitors, was surprising as previously it had been reported that Pompe leucocytes had little activity with glycogen as substrate (Koster et al., 1974), whereas considerable activity remained in Pompe kidney (Koster et al., 1976). Previously, Salafsky and Nadler (1971) had noted that the maltase activity of amniotic fluid differed from that of kidney in heat stability.

(iv) Diagnosis of Pompe's disease in lymphocytes and in leucocytes after pH 5.0 precipitation.

Lymphocyte α-glucosidase responded to inhibitors in the same way as that of control liver. This was in agreement with Koster et al. (1974), who claimed that lymphocytes, but not dextran isolated leucocytes, were a suitable material for the detection of Pompe's disease.

Much of the α-glucosidase of dextran isolated leucocytes was removed by pretreatment at pH 5.0 followed by centrifugation. The remaining activity assayed at pH 4.0 was almost all acid α-glucosidase as demonstrated with inhibition studies using turanose, maltose and citrate. This suggests that pH 5.0 precipitation is of use for the diagnosis of Pompe's disease in leucocytes. The resuspended pH 5.0 precipitate was inhibited by turanose and maltose as for Pompe kidney. The results for citrate were anomalous; the explanation was that the small amount of phosphate (4mM final in the assay) was sufficient to modify the inhibitory effect of citrate (0.2M) considerably.

It is concluded that Pompe's disease may be diagnosed using dextran isolated leucocytes either following pH 5.0 precipitation or by inhibition studies, but that lymphocytes are a much better diagnostic material.

# PART 3

## β-D-GLUCOSIDASE

## AND ITS

## DEFICIENCY IN GAUCHER'S DISEASE

GAUCHER'S DISEASE AND ITS ASSOCIATED ENZYME DEFICIENCY.

The inherited disorder now known as Gaucher's disease was first described by Gaucher (1882). The most common form of the disease is adult or chronic non-neuronopathic Gaucher's disease and, as it was the first to be described is known as type 1. Adult Gaucher's disease is particularly frequent in Jewish populations. The infantile and juvenile forms, which have neurological involvement, are less common. There is overlap in severity within both the non-neuronopathic form of the disease and the neuronopathic forms. The safest classification would seem to be based on whether or not there is neurological involvement. Of the six cases encountered, five were neurologically affected. This was probably a reflection of the genetic pool of Scotland and the age of patients referred to the laboratory for enzyme studies.

Lieb (1924), investigating the splenomegaly found in Gaucher patients, isolated large amounts of cerebrosides from spleen. At first it was thought that this was galactocerebroside but Klenk and Harle (1930) observed that the cerebroside isolated from Gaucher spleen differed from galactocerebroside in optical rotation. Aghion (1934) showed that it was in fact the glucosyl ceramide and not the galactosyl ceramide which accumulated in Gaucher spleen. With interest in the lysosomal storage disorders arising from the discovery that Pompe's disease was due to a deficiency of lysosomal α-1,4-glucosidase (Hers, 1963), it seemed likely that Gaucher's disease was due to a deficiency of the enzyme responsible for the catabolism of glucosyl ceramide.

This was indeed the case and a deficiency of glucosyl ceramide β-glucosidase activity was demonstrated in Gaucher tissues (Brady et al., 1965; Patrick, 1965).

Patrick (1965) demonstrated the deficiency in Gaucher spleen by measuring the rate of release of glucose from glucosyl ceramide, the natural substrate of the enzyme. Using the artificial substrate, p-nitrophenyl-β-D-glucoside, the β-glucosidase activity of all Gaucher spleens tested was less than 20% of the control mean (Patrick, 1965). He also found that liver from Gaucher patients had 30% of the control activity when glucosyl ceramide was used as substrate, but that the p-nitrophenyl-β-D-glucosidase activity was twice the control level! Using another artificial substrate, 4-methylumbelliferyl-β-D-glucopyranoside, Öckerman (1968) found one peak of hepatic β-glucosidase on Sephadex G-150 gel filtration and that this peak was coincident with that of β-xylosidase and the final peak of β-galactosidase. In Gaucher's disease, β-glucosidase, β-xylosidase and the final peak of β-galactosidase were deficient and it was suggested that all three activities were attributable to the same enzyme protein. Butterworth et al. (1972) obtained two peaks of β-glucosidase activity, one of which was in the void volume and the other corresponding to that of Öckerman (1968). Using spleen, Ho (1973) obtained two peaks of activity, but she had to incubate the homogenate in water for one hour at 24°C in order to solubilise the first peak and so prevent its loss on centrifugation at 100 000g. She also showed that the glucosyl ceramide β-glucosidase was

associated with the first peak of non-specific β-glucosidase but not with the second peak. This was in agreement with earlier work (Ho and O'Brien, 1971a) demonstrating that the deficiency in Gaucher's disease was associated with the particulate β-glucosidase activity and not the soluble. However, Kanfer et al. (1975) have claimed that both the particulate and soluble β-glucosidases may be deficient in Gaucher's disease. The nature of the deficiency in Gaucher liver using 4-methylumbelliferyl-β-D-glucopyranoside as substrate was investigated in this thesis.

Apart from the apparent inconsistencies outlined above, it was still likely that one enzyme corresponded to more than one hydrolase activity. There was the component of β-glucosidase, which also had β-galactosidase and β-xylosidase activity and was reported to be deficient in Gaucher's disease, corresponding to peak III of β-galactosidase on Sephadex G-150 gel filtration (Öckerman, 1968). The three β-galactosidase peaks of Öckerman (1968) had pH optima of 4.1, 4.7 and 5.3 respectively. This was interesting because it had been claimed (O'Brien et al., 1972) that in $G_{M1}$ gangliosidosis type 1, liver homogenates were deficient in all β-galactosidases, whereas in type 2 the component with highest pH optimum remained. The deficiency of neutral β-galactosidase in type 1 $G_{M1}$ gangliosidosis has, however, been questioned (Suzuki and Suzuki, 1974b). Other data is just as confusing. α-Arabinosidase and β-fucosidase were reported to be deficient in tissues from patients who had died of $G_{M1}$ gangliosidosis (van Hoof and

Hers, 1968; Hindman and Cotlier, 1972). Liver β-glucosidase was shown to be indistinguishable from one of the β-galactosidase components by gel filtration, starch gel electrophoresis, heat stability and its response to the presence of chloride ions (Ho and O'Brien, 1971b). However, owing to the speed of centrifugation used, they were probably only investigating the soluble component of β-glucosidase. Norden et al. (1974) claimed that another component of β-galactosidase also had β-fucosidase and α-arabinosidase activity, whereas previously Hultberg et al. (1973) had reported that Gaucher fibroblasts were deficient in α-arabinosidase as well as β-xylosidase and β-glucosidase. More recently, Chester et al. (1976) assigned five separate glycosidase activities to the same enzyme molecule. They eluted two peaks of liver β-galactosidase from DEAE-cellulose, the first of which had an optimum of pH 5.5 and also had β-glucosidase, β-fucosidase, β-xylosidase and α-arabinosidase activities. Some of the α-arabinosidase activity was also associated with peak II. When the fractions were reassayed after storage at 4°C for four weeks, there was little diminution of any of the peak I activities. The β-galactosidase activity in peak II, however, had been totally lost, whereas about half the α-arabinosidase activity remained. This was presented as evidence that the acid β-galactosidases were not identical with the coincident α-arabinosidase. Furthermore it was shown using mixed substrate competition experiments, that peak III of β-galactosidase on Sephadex G-150 was identical with β-glucosidase as indeed were β-xylosidase, α-arabinosidase and β-fucosidase. Some of these claims will be looked at in the light

of the enzyme deficiencies in tissues of patients who died of $G_{M1}$ gangliosidosis and Gaucher's disease.

The enzymic diagnosis of Gaucher's disease has been achieved using leucocytes (Kampine et al., 1967; Beutler and Kuhl, 1970) and fibroblasts (Beutler et al., 1970). It was found (Beutler and Kuhl, 1970) that leucocytes had a double peak of 4-methylumbelliferyl-β-D-glucosidase activity with optima at pH 4.0 and pH 5.3 and that Gaucher leucocytes lacked the peak with optimum, pH 4.0. Cultured skin fibroblasts had only a single peak of activity with an optimum of pH 4.0-4.5 (Beutler et al., 1971).

Ho et al. (1972) reported that fibroblast β-glucosidase activity was not linear with dilution and it was proposed that this was due to the dilution of an important co-factor of the enzyme. This co-factor was not lost by ultrafiltration and it was suggested that this might be a protein of molecular weight greater than 1000. For the assay of β-glucosidase these workers used water homogenates and the pH profile for fibroblasts had a broad optimum of pH 4.5-5.7. This was in marked contrast to the sharp optimum obtained with saline suspended cells (Beutler et al., 1971). An interesting finding was the stimulation of β-glucosidase activity using 0.02% Triton X-100 (Ho et al., 1972). At Triton X-100 concentrations lower than 0.02%, no activation occurred and at higher concentrations the enzyme was inhibited. When Gaucher fibroblasts were tested, no stimulation occurred. These results were at variance with those of Beutler et al. (1971) who found no stimulation of β-glucosidase activity at concentra-

tions of Triton X-100 between 0.004% and 0.25%.

The non-linearity of acid β-glucosidase on dilution, which was discussed earlier, suggested that the enzyme activity might be dependent on more than one constituent. In support of this, it was found that spleen homogenates from a control and a case of adult Gaucher's disease, when mixed, had 2-3 times the theoretical expected enzyme activity (Ho and O'Brien, 1971a). These workers were also able to separate two factors, namely P and C, both of which were important for maximum activity. It was proposed that factor C was the catalytic protein, eluting with peak I β-glucosidase on Sephadex G-150 gel filtration and that factor P was an essential activator of factor C. The level of factor P extracted from adult Gaucher spleen was 10-17 times greater than that from control spleen and this led to the suggestion that this disease was attributable to a deficiency of factor C. Ho (1973) went on to show that glucocerebrosidase activity, as well as the acid β-glucosidase, was also dependent on the association of factors P and C. Furthermore, it was shown that the glucocerebrosidase and β-glucosidase activities of factor C, solubilised with 0.1% Triton X-100, could be stimulated using 0.1% sodium taurocholate. Another important requirement for maximum enzyme activity was found to be acidic membrane phospholipids, addition of which to factor C increased activity greatly (Ho and Light, 1973) particularly in the presence of factor P. The loss of activity when factor C was solubilised (Ho and O'Brien, 1971a) was attributable to its removal from the cell membrane and consequently its separation from these phospholipids. In this thesis the effects of

Triton X-100, sodium taurocholate and phosphatidylserine were investigated for control and Gaucher fibroblast 4-methylumbelliferyl-β-D-glucosidases.

METHODS.

Enzyme assay.

β-Glucosidase was assayed using 4-methylumbelliferyl-β-D-glucopyranoside (1mM). The activity at pH 4.0 was used for diagnostic purposes, but usually diagnosis was undertaken by pH profile. Column fractions were assayed at pH 5.0. For assays at a single pH, substrate (2mM), dissolved in acetate buffer (0.2M) containing 0.02%$^{w}$/v sodium azide, was mixed with an equal volume of extract. After incubation at 37°C for a known time (usually 60 minutes), the reaction was stopped by the addition of 0.1M sodium carbonate and the fluorescence read as described in the appendix (page 242). For pH profiles, substrate (4mM) dissolved in distilled water was added to an equal volume of acetate buffer (0.4M) containing 0.02%$^{w}$/v sodium azide and the assay was started with extract (2 volumes).

In experiments where the assay was carried out in the presence of sodium taurocholate (0.1%$^{w}$/v), this was included when the buffer was made up. Triton X-100 and phosphatidylserine were included in the assay by reducing the volume of extract and making up the volume with either distilled water for the control experiment or additive, e.g. 25µl buffer + 25µl substrate + 10µl distilled water or Triton X-100 (ten times final concentration) + 40µl extract.

α-L-Arabinosidase, β-D-fucosidase, β-D-galactosidase and β-D-xylosidase were assayed using the appropriate 4-methylumbelliferyl conjugate, final concentration, 0.5mM. All these hydrolase

activities were assayed in acetate buffers in the absence of Triton X-100. The β-xylosidase assay was incubated for six or seven hours and read immediately after adding alkali, but a shorter time, usually an hour, was sufficient for the other hydrolase activities and the reading was stable after the addition of alkali. Protein was estimated (Lowry et al., 1951) as described in the appendix (page 243).

Enzyme source and preparation of extract.

(i) Cultured cells.

Amniotic fluid cells and skin fibroblasts were cultivated as described in the appendix (page 244).

(ii) Leucocytes.

Leucocytes were separated from whole blood by differential sedimentation in dextran as described in the appendix (page 247).

(iii) Post-mortem tissues.

Tissues, liver and spleen, were stored at -40°C until required.

Cultured cells and leucocytes were usually suspended in distilled water using a vortex mixer before assay. Such homogenates were not usually sonicated nor centrifuged. For special studies, cells were sometimes suspended in isotonic saline and dispersed once again using a vortex mixer. Occasionally, saline suspended cells, particularly leucocytes, were difficult to disperse owing to clumping and this was overcome by squirting through a syringe needle a couple of times.

Tissues were homogenised using a Potter-Elvehjem all glass hand homogeniser. For gel filtration, homogenates (10%$^{w}$/v) were prepared in elution buffer and centrifuged at 900g for fifteen minutes. For other studies, homogenates (1 or 2% $^{w}$/v) were prepared in the appropriate extraction medium and larger debris allowed to settle for three minutes. The supernatant was carefully removed for assay. When the effects of different concentrations of EDTA and sodium chloride were tested, the liver was first homogenised in distilled water and then diluted 1:1 with double the concentration of EDTA or sodium chloride required. When extracts were sonicated, a "Soniprobe" (Dawe's Instruments) was used.

Gel filtration.

Ascending gel filtration was carried out at 4°C using a column (35 x 3cm diam.) of Sephadex G-150 in 10mM-sodium phosphate elution buffer, pH 6.0, containing 10mM-EDTA and 400mM-NaCl. A flow rate of 4.5ml/hr was used and 1.5 ml fractions collected for assay.

(a) STUDIES ON HUMAN NON-SPECIFIC β-GLUCOSIDASES.

RESULTS.

(i) Separation of β-glucosidase components on Sephadex G-150.

Sephadex G-150 gel filtration of normal human liver extracts, prepared by homogenisation in a Potter-Elvehjem all glass homogeniser followed by low speed centrifugation, usually gave two peaks of β-glucosidase activity, the first of which was in the void volume (Figure 58a, page 153). On one occasion the second peak of activity was missing, although there was no suggestion of Gaucher's disease. It was thought possible that sonication of the extract might release a soluble component from the void volume peak. In order to check this, homogenised extracts, which had either been sonicated or not treated further, were run consecutively on the same column, but it was found that no extra peak was generated (Figure 58b, page 153).

Two peaks of β-glucosidase activity were also obtained with spleen homogenates, although the second peak was much smaller relative to the void volume peak than was usually found for liver (Figure 59a, page 154). Homogenates of cultured skin fibroblasts, cultured amniotic fluid cells and leucocytes were also investigated by gel filtration (Figures 59b,c,d, page 154). Although the activity of these homogenates was low, a recognisable first peak of activity was detected but there was no second peak. It was possible that such a peak was there, but that its activity fell below the sensitivity of the assay. Nevertheless, it would seem

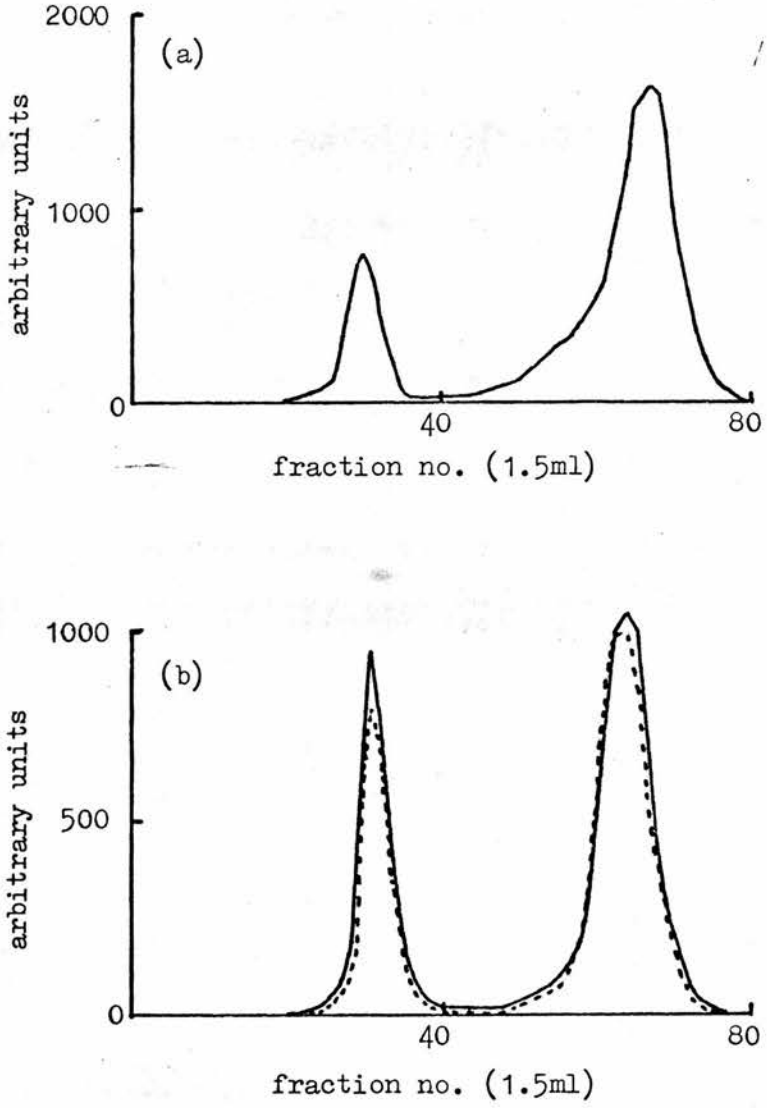


FIGURE 58. (a) Sephadex G-150 gel filtration of control liver β-glucosidase.

(b) Comparison of Sephadex G-150 gel filtration profiles of β-glucosidase from hand-homogenised (———) and sonicated (- - - - - -) control liver.

that all or nearly all the β-glucosidase activity in cultured cells and leucocytes was associated with the void volume peak.

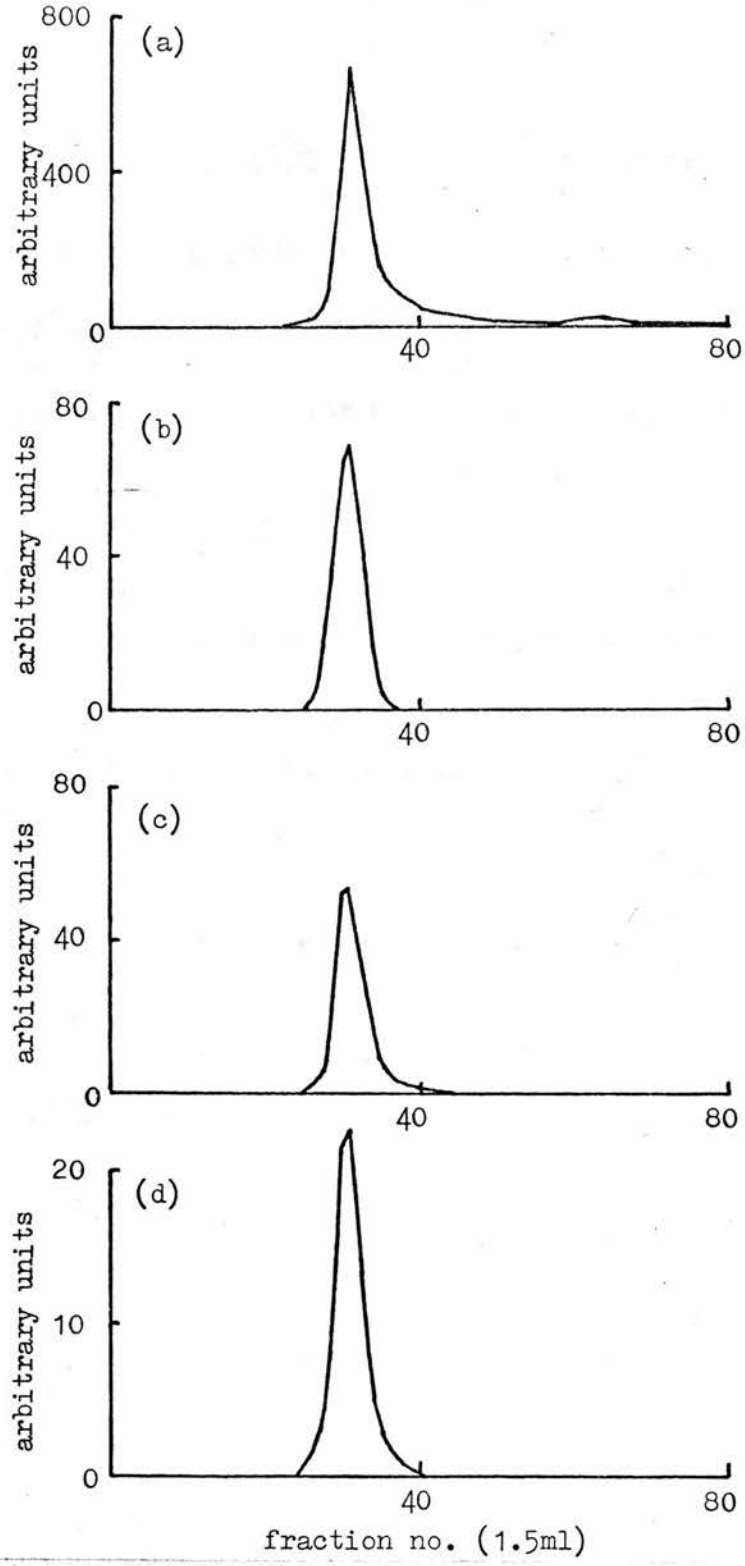


FIGURE 59 . Sephadex G-150 gel filtration of β-glucosidase from (a) spleen, (b) cultured skin fibroblasts, (c) cultured amniotic fluid cells and (d) leucocytes.

(ii) pH profiles of β-glucosidases from different tissues.

The pH profiles for total water homogenates of liver, spleen, leucocytes and cultured cells are shown in Figure 60 . Liver and spleen β-glucosidase profiles were similar with an optimum of pH 4.5-5.5. Although control fibroblasts had a broad optimum

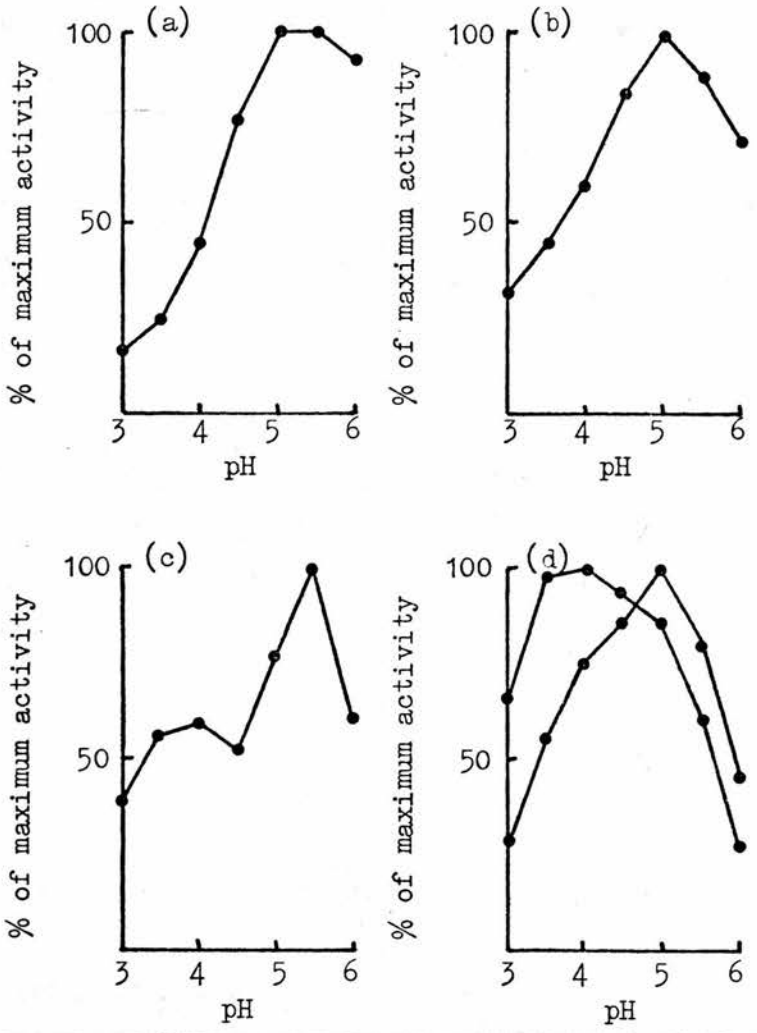


FIGURE 60. pH profiles of β-glucosidase from water homogenates of (a) liver, (b) spleen, (c) leucocytes and (d) fibroblasts (two examples).

between pH 3.5 and pH 5.0, this could be skewed either way in different cell homogenates; two examples with different profiles are shown in Figure 60d, page 155). Leucocyte β-glucosidase was more active at higher pH values and as with fibroblasts seemed to consist of more than one component.

The pH profiles of the peaks from the Sephadex G-150 column were investigated to see which part of the total activity they represented. The results (Figure 61 ) were quite unexpected. Peaks I and II from liver differed considerably. Peak I pH profile was fairly symmetrical with a sharp optimum at pH 5.0. Peak II, on the other hand, had a flattish optimum of pH 5.5-6.0 with very little activity at pH 3.0-4.0. The combined activity of the two peaks at pH 4.0 was much lower than would have been predicted using total water homogenates. This difference was

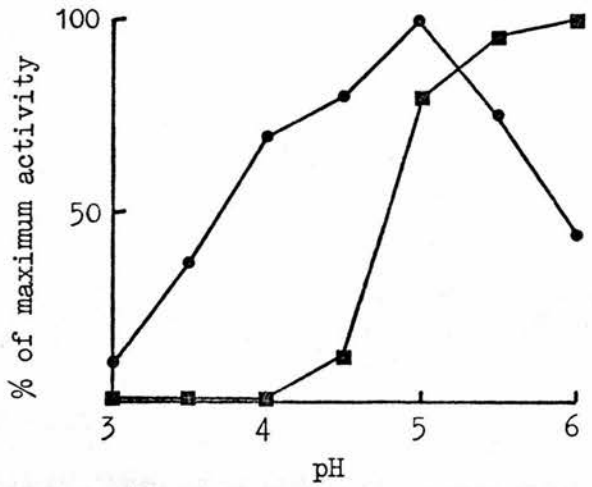


FIGURE 61. pH profiles of β-glucosidase for peak I (•) and peak II (■) from Sephadex G-150 gel filtration of control liver.

later shown (page 182) to be a consequence of the presence of constituents of the elution buffer in the assay. The single peak of β-glucosidase activity obtained by Sephadex G-150 gel filtration, for cultured skin fibroblasts and leucocytes (Figure 62), had an optimum at pH 5.0, but in each case the optimum was less sharp than for liver peak I. Although the profiles for cultured skin fibroblasts and leucocytes were similar, the optimum skewed towards the acid side for fibroblasts and towards the neutral for leucocytes.

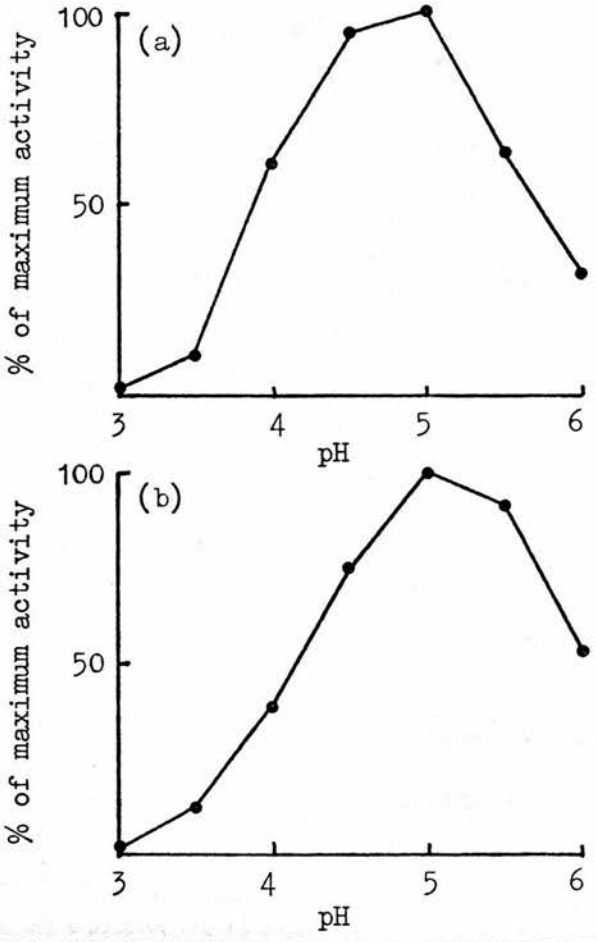


FIGURE 62. pH profile of the peak of β-glucosidase activity eluted by Sephadex G-150 gel filtration of (a) cultured skin fibroblasts and (b) leucocytes.

(iii) Factors affecting the β-glucosidase pH profile of cultured cells.

The effect on the pH profile of β-glucosidase of assaying in the presence of Triton X-100 (0.02%$^{V}$/v) and/or sodium taurocholate (0.1%$^{W}$/v) was tested. A typical result is shown in Figure 63 . In the presence of Triton X-100, the usual broad optimum was

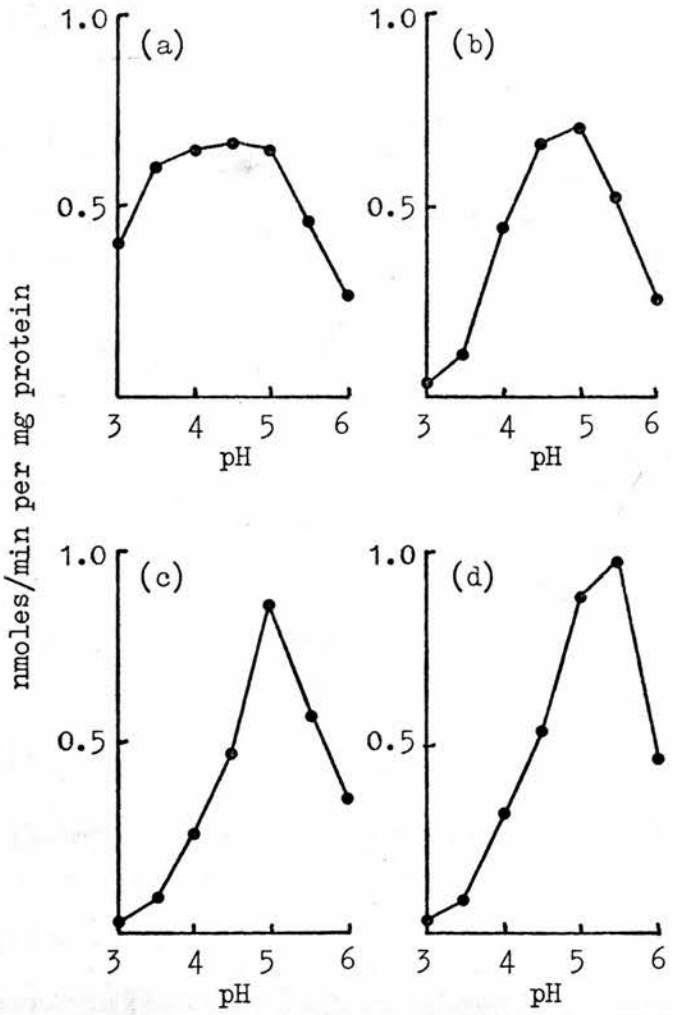


FIGURE 63. Comparison of pH profiles of fibroblast β-glucosidase assayed (a) with no addition, (b) with 0.1% $^{W}$/v sodium taurocholate, (c) with 0.02% $^{V}$/v Triton X-100 and (d) with 0.02% $^{V}$/v Triton X-100 and 0.1% $^{W}$/v sodium taurocholate.

transformed to a sharp peak at pH 5.0. In the example, activation at pH 5.0-6.0 did occur but this was not always found. The most dramatic effect was the reduction in activity at acid pH particularly at pH 3.5. Sodium taurocholate had a similar effect to Triton X-100, but the new optimum although at pH 5.0 was not as sharp. A combination of sodium taurocholate and Triton X-100 gave an optimum at pH 5.5 and invariably activated at that pH.

To check that the critical Triton X-100 concentration used by Ho (1972) had not been missed, the activity of fibroblast β-glucosidase at pH 4.0 in concentrations of Triton X-100 from 0 to 0.02%$^{v}$/v was determined. It can be seen in Figure 64 that 20-25% activation was achieved for concentrations between 0.002% and 0.010% $^{v}$/v Triton X-100. The activity at a concentration of 0.02%$^{v}$/v Triton X-100 was only 10% of the activity when none was

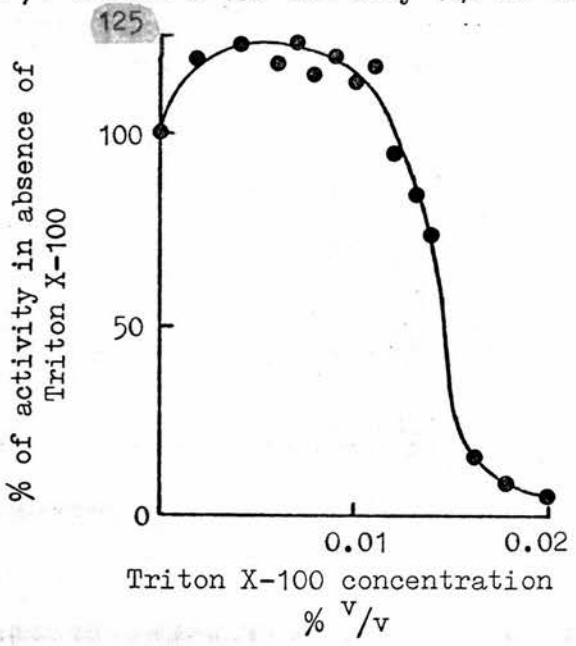


FIGURE 64. Effect of Triton X-100 concentration on fibroblast β-glucosidase activity at pH 4.0.

used. Any further studies in this section in which Triton X-100 was used were standardised at a concentration of 0.006%$^{v}$/v.

The rôle of phosphatidylserine (Ho and Light, 1973; Ho and Rigby, 1975) in the association of factors P and C raised the question of what effect this acidic phospholipid would have on fibroblast β-glucosidase activity. Once again an unexpected result was obtained (Figure 65 ). The typical broad pH optimum for fibroblasts of pH 3.5-5.0 was altered to a sharp optimum at pH 5.0-5.5 in the presence of 0.15mM phosphatidylserine. In the example, the activity at pH 4.0 was inhibited by 88% and at pH 5.5 was activated by 46%.

From the diagnostic point of view, it was claimed that

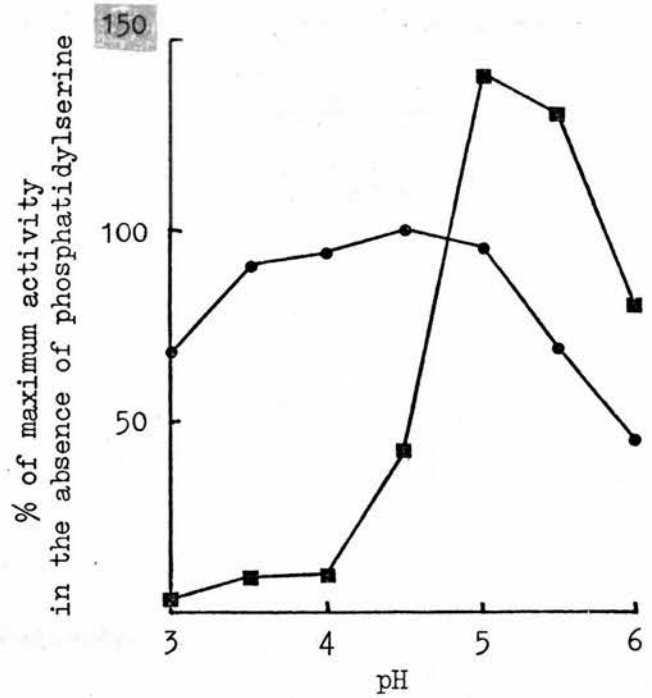


FIGURE 65 . Effect of 0.15mM phosphatidylserine on the pH profile of fibroblast β-glucosidase

(•) with no addition (■) with phosphatidylserine

optimal conditions for assay of β-glucosidase was with cells suspended in isotonic saline (Beutler et al., 1971). This was of interest because it became apparent that the peaks of β-glucosidase activity eluted from the Sephadex G-150 column had been inhibited at acid pH in the presence of sodium chloride (page 183 ). The effect of sodium chloride on β-glucosidase was investigated for cultured amniotic fluid cells and skin fibroblasts. The cells were assayed after preparation by three different methods; cells were lysed in water, suspended in isotonic saline or lysed with one volume of water for five minutes and brought back to isotonicity with one volume of double strength saline. Each of these methods gave rise to different results (Figure 66 , page 162 ). The specific activity at pH 4.0 was usually greatest in the case of saline suspended cells, although the activity at pH 3.0 was greatly reduced. The overall effect of using saline suspended cells was that the optimum was sharpened to pH 4.0-4.5. Addition of saline to lysed cells did not increase the activity at pH 4.0 but severely inhibited the enzyme at pH 3.0-3.5, whilst the activity at pH 5.0-6.0 was intermediate between that of saline suspended and water lysed cells. The effect of sodium chloride seemed to be dependent to some extent on the integrity of the cell, but inhibition at pH 3.0-3.5 always occurred.

Another approach was to assay saline suspended cells in the presence of Triton X-100 (0.006%$^{v}$/v) and/or sodium taurocholate (0.1%$^{w}$/v). It should be noted that the concentration of Triton X-100 was 0.006%$^{v}$/v and not 0.02%$^{v}$/v as used previously. The results (Figure 67 , page 163 ) once again showed the shift to a

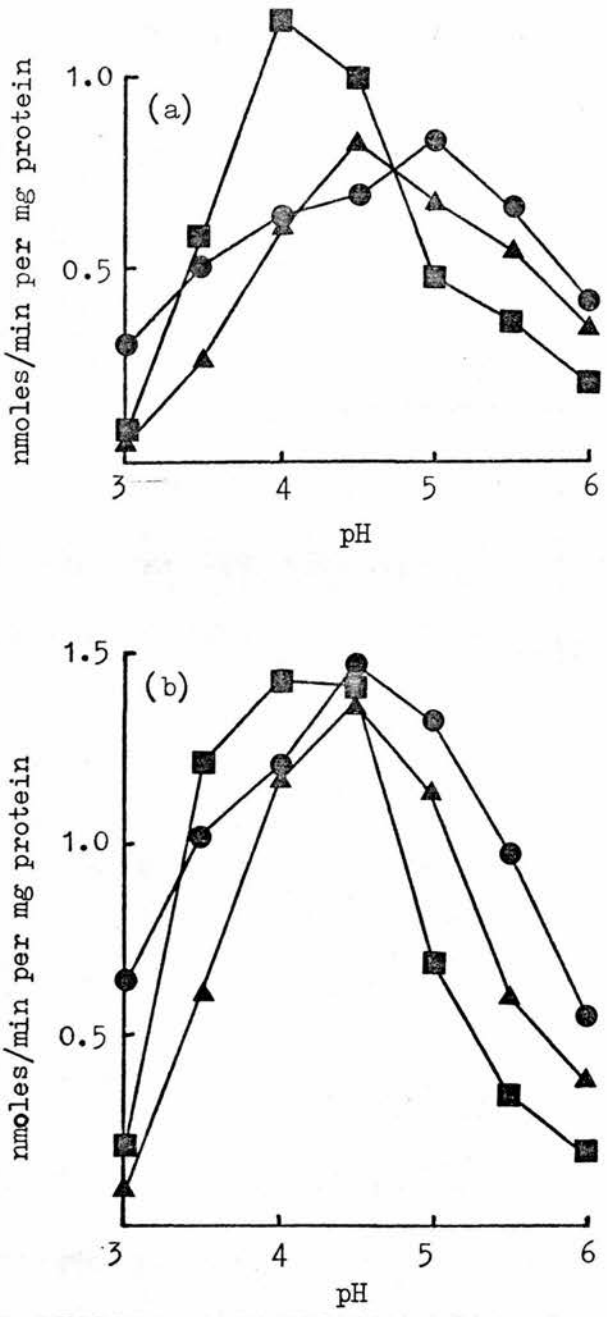


FIGURE 66. Effect of sodium chloride on the β-glucosidase pH profile of (a) amniotic fluid cells and (b) skin fibroblasts.

(●) cells lysed in distilled water

(■) cells suspended in isotonic saline

(▲) cells lysed in distilled water then brought back to isotonicity with sodium chloride.

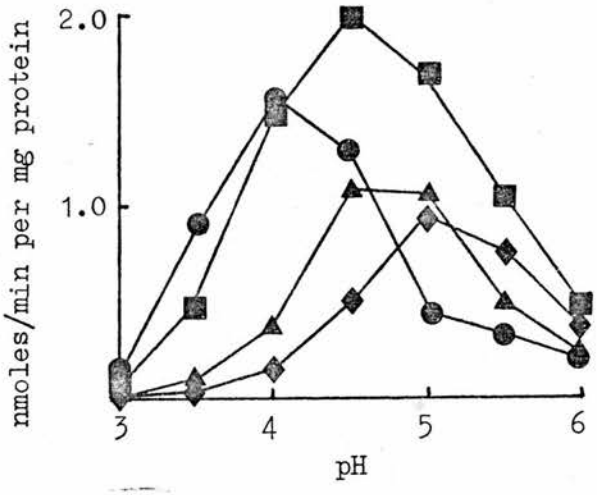


FIGURE 67. Effect on the β-glucosidase pH profile of saline-suspended cultured cells of 0.006% $^{v}/_{v}$ Triton X-100 (■), 0.1% $^{w}/_{v}$ sodium taurocholate (▲) and 0.006% $^{v}/_{v}$ Triton X-100 with 0.1% $^{w}/_{v}$ sodium taurocholate (◆) compared with no addition (●).

higher pH optimum in the presence of sodium taurocholate or Triton X-100. The most dramatic effect was the marked stimulation of β-glucosidase activity at pH 4.5-5.0 when cells suspended in isotonic saline were assayed in the presence of 0.006%$^{v}/_{v}$ Triton X-100.

Figure 68 (page 164) shows the effect of centrifugation and Figure 69 (page 164), the effect of centrifugation following sonication on the β-glucosidase pH profile of water lysed cells. Although the three cultures (Figure 68 , page 164 ) differed slightly in pH profile, in each the total and precipitated activities were very similar. A fourth culture was divided into two halves and one half subjected to sonication before centrifugation, resulting in a quite different result (Figure 69 , page 164). The sedimented fraction no longer resembled the total activity in pH profile. The sonicated total had a different pH profile from the

unsonicated total and there was some loss of activity at all pH values. Sonication markedly increased the β-glucosidase activity in the supernatant.

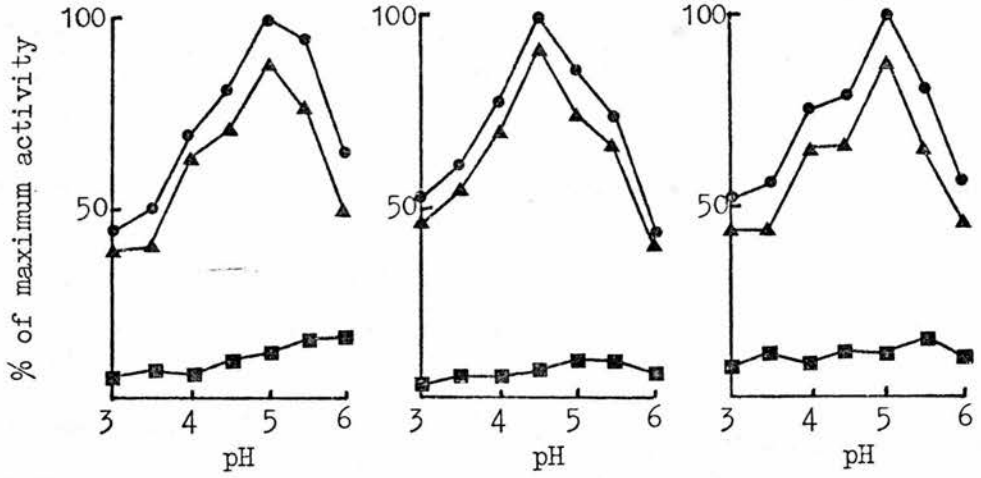


FIGURE 68. Effect of centrifugation at 25 000g on the β-glucosidase pH profile of cultured cells (3 examples).

(▲) precipitate (■) supernatant (●) total

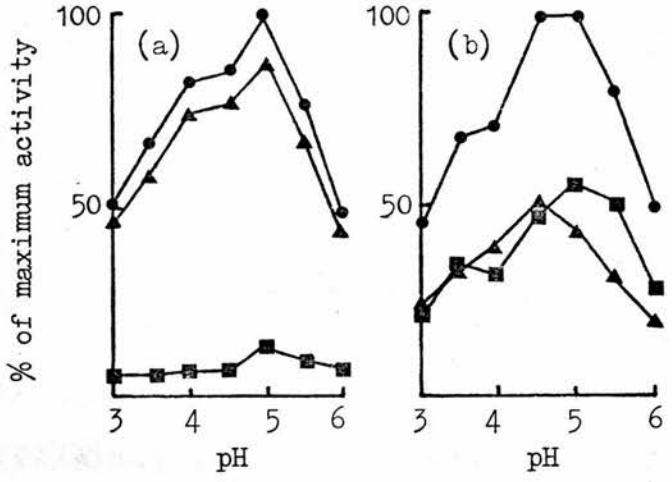


FIGURE 69. Comparison of pH profiles of cultured cells, (a) unsonicated and (b) sonicated prior to centrifugation at 25000g.

(▲) precipitate (■) supernatant (●) total

(iv) The association of β-glucosidase with other glycosidase activities.

It has been claimed (Öckerman, 1968; Chester et al., 1976) that β-glucosidase was associated with β-xylosidase, β-fucosidase and the small molecular weight components of β-galactosidase and α-arabinosidase. The Sephadex G-150 elution profile for normal human liver is shown in Figure 70 (page 166 ) when assayed for the five enzymes, β-galactosidase, β-xylosidase, β-fucosidase, α-arabinosidase and β-glucosidase. It was observed that each had activity at or just after the void volume and that all activities were also detected in the final peak, which eluted just before hæmoglobin. Other components of β-galactosidase, β-fucosidase and α-arabinosidase eluted between these two peaks. Using this crude system, it was possible to separate some of the β-galactosidase, β-fucosidase and α-arabinosidase components from β-glucosidase and β-xylosidase.

The pH profile for β-xylosidase in control cultured skin fibroblasts was very similar to the pattern for β-glucosidase and a sharpening of the optimum to pH 4.0-4.5 was observed when cells were suspended in saline before assay (Figure 71 , page 167 ). Similarly, the pH profiles of fibroblast β-fucosidase and α-arabinosidase were alike, but differed from β-glucosidase (Figure 72, page 167). Unexpectedly, fibroblast β-galactosidase differed from both β-fucosidase and α-arabinosidase in pH profile (Figure 73 , page 168 ).

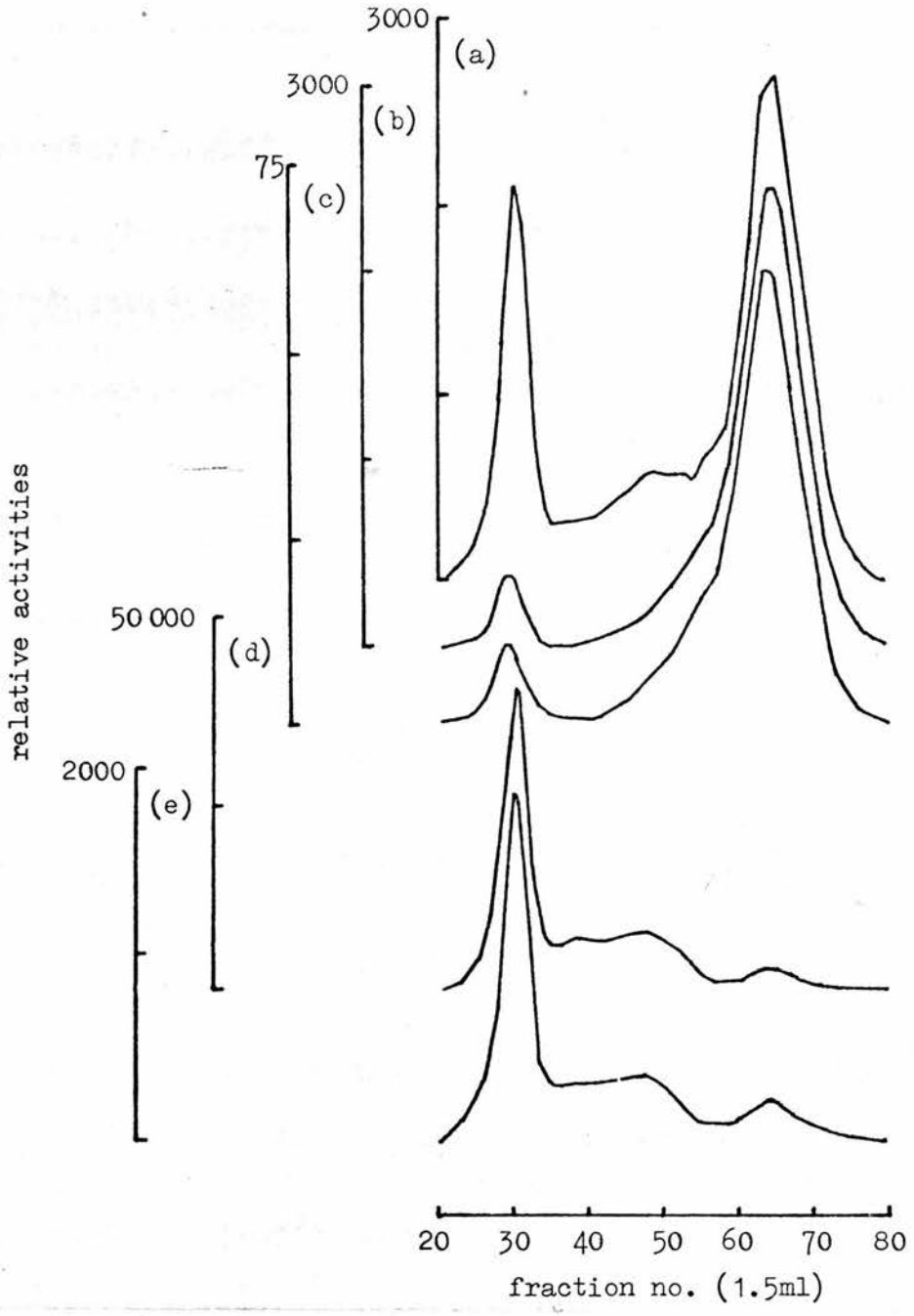


FIGURE 70. Sephadex G-150 gel filtration of (a) β-D-fucosidase, (b) β-D-glucosidase, (c) β-D-xylosidase, (d) β-D-galactosidase and (e) α-L-arabinosidase.

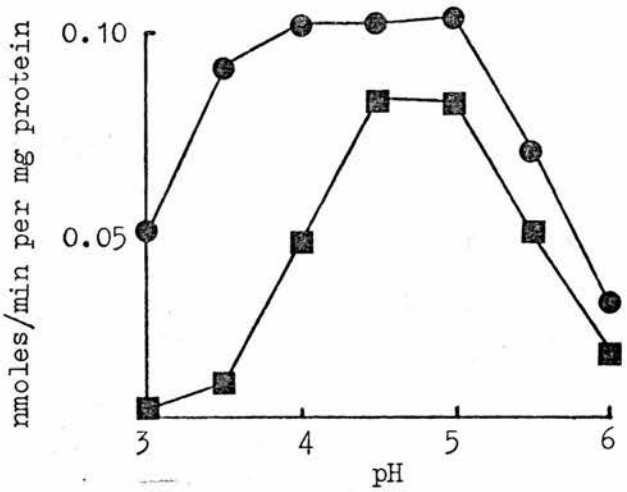


FIGURE 71. β-xylosidase pH profile of cultured skin fibroblasts lysed in distilled water (●) or suspended in saline (■) prior to assay.

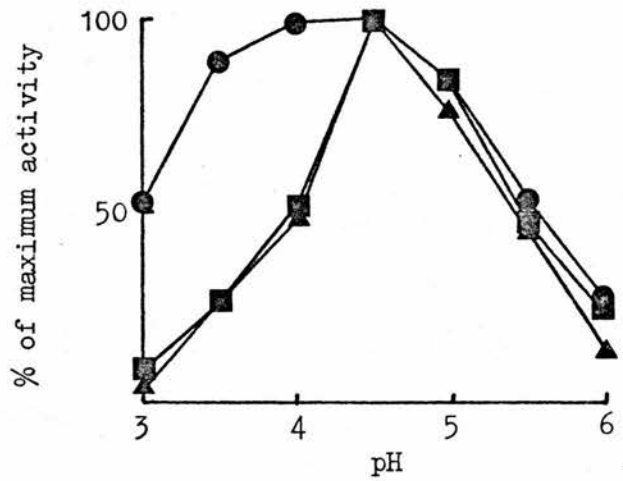


FIGURE 72. Comparison of the pH profiles of fibroblast β-D-glucosidase (●), β-D-fucosidase (■) and α-L-arabinosidase (▲).

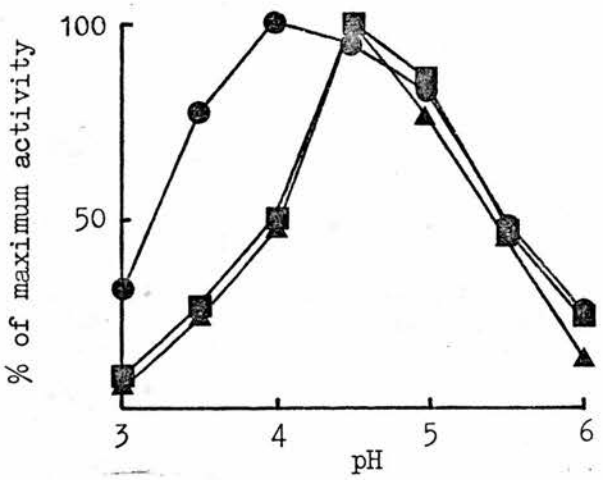


FIGURE 73. Comparison of the pH profiles of fibroblast β-D-galactosidase (●), β-D-fucosidase (■) and α-L-arabinosidase (▲).

DISCUSSION.

(i) Gel filtration of β-glucosidases.

Non-specific β-glucosidases were separated into two peaks by Sephadex G-150 gel filtration. Previously, Öckerman (1968) had detected only one peak of β-glucosidase activity. The methods used had been very similar except that the pH of the elution buffer used here was pH 6.0 not pH 7.6 and the homogenate had not been centrifuged at 100 000g. In view of the known membrane-bound nature of β-glucosidase, a low speed spin to remove large particles only was used. Butterworth et al. (1972) also obtained two peaks of activity having centrifuged homogenates at 25 000g. It was likely, therefore, that Öckerman had completely lost one of the β-glucosidase peaks in his procedure for homogenate preparation. Although the method employed here was not ideal owing to the membrane-bound nature of the enzyme, it was possible to isolate more than one component. The ineffectiveness of sonication on the elution profile suggested that the peak I enzyme eluted in the void volume irrespective of the degree of disruption of the membrane. Ho (1973) was able to solubilise some of the membrane-bound β-glucosidase of spleen by incubation in water for one hour at 24°C and was able to show that the enzyme, which was not now removed by centrifugation at 100 000g, was eluted in the void volume. It was also shown that this peak I β-glucosidase corresponded to glucocerebrosidase whereas peak II had no associated glucocerebrosidase activity. The finding (Öckerman, 1968), that homogenates of Gaucher liver were deficient

in soluble β-glucosidase remains puzzling. One liver homogenate encountered in this study had no peak II activity and this was also found in previous studies (Butterworth et al., 1972). It is possible that peak II is a fossil enzyme and now has no function in which case a deficiency would confer no particular disadvantage. It does not seem likely however that all the Gaucher livers used by Ockerman were by chance deficient in this enzyme whereas all the controls had the soluble β-glucosidase.

The β-glucosidase activity of cultured cells and leucocytes was eluted in the void volume which is consistent with the use of these cell types in diagnosis (Beutler et al., 1971).

(ii) pH profiles of β-glucosidases.

Although the β-glucosidase activity of leucocytes and cultured cells was excluded from Sephadex G-150, its pH profile was different for the two cell types when assayed using water homogenates. Whereas leucocytes appeared to be composed of two components, the more neutral of which had the greater activity, the situation in cultured cells was less easy to interpret. In view of the different profiles obtained for different cell cultures, it was likely that here again more than one component was present. The difference between the two appeared to be that leucocytes had more of the neutral β-glucosidase, whereas the predominant component in cultured cells was the acid β-glucosidase. Changes in the proportion of the acid and neutral components may give rise to the variations observed in the pH profiles of cultures.

When the β-glucosidase pH profiles of cultured cells and leucocytes, eluted from the Sephadex G-150 column, were investigated they were very much alike. There was still a suggestion, however, that cultured cells had more of the acid component than leucocytes. These profiles were not unlike that of peak I β-glucosidase from liver which also appeared to have more than one component. The relative activities of these components was also probably responsible for differences in profiles.

(iii)Effects of Triton X-100, sodium taurocholate and phosphatidylserine on β-glucosidase activity.

The sensitivity of β-glucosidase to the conditions of assay almost certainly reflects the importance of an association between enzyme and membrane for function. It was not possible to obtain the same effect using Triton X-100 as Ho (1972). Activation at pH 4.0 was only obtained at concentrations of Triton X-100 below 0.010%$^{v}$/v and even at these concentrations, the stimulation was not as great as had been reported. Nevertheless, activation did occur using 0.02%$^{v}$/v Triton X-100 at pH 5.0-6.0 on some occasions. Similar results were obtained using sodium taurocholate. The shift in pH optimum which occurred when Triton X-100 or sodium taurocholate was included in the assay may have been due to a change in the environment of the enzyme or possibly the differential stimulation of one β-glucosidase component coincident with the inhibition of another. Similarly, the effect of phosphatidylserine on fibroblast β-glucosidase was either to convert all the activity to a form with an optimum of pH 5.0-5.5 or to activate that component with an optimum at pH 5.0-5.5 and, at the same time, inhibit the acid β-glucosidase.

(iv) Effect of sodium chloride on β-glucosidase activity.

Suspending fibroblasts in isotonic saline often resulted in activation of β-glucosidase at pH 4.0-4.5 and inhibition on either side of this narrow pH range. The major contributory factor to this effect was the maintenance of cellular structure. This was demonstrated when cells were lysed before the addition of sodium chloride. The effect of Triton X-100 on saline suspended cells at pH 3.0-3.5 was not unexpected. The release of the enzyme from the cell left it susceptible to inhibition by sodium chloride. The activation at pH 4.5-5.0 was more surprising and is unexplained.

(v) Centrifugation of the β-glucosidase of cultured cells.

When cultured cells were lysed in distilled water and centrifuged at 25 000g, there was no evidence that the small fraction of the β-glucosidase activity in the supernatant differed significantly from that in the sediment. This supported the finding with Sephadex G-150 gel filtration that cultured cells had no low molecular weight β-glucosidase. If, however, the cell extract was sonicated before centrifugation, a much larger proportion of the β-glucosidase was found in the supernatant. Furthermore, the β-glucosidase pH profiles of the sediment and the supernatant were quite different. This again supported the idea that the variability of profiles was dependent on the environment of the enzyme when assayed.

(vi) The common identity of β-glucosidases with other glycosidases.

From the studies comparing several hepatic glycosidase activities, no evidence was obtained to show that the final peak of β-galactosidase on Sephadex G-150 gel filtration was not the same enzyme molecule as β-glucosidase, β-fucosidase, β-xylosidase and α-arabinosidase eluting in the same fractions, as proposed by Öckerman (1968). It also became apparent that the other components of β-fucosidase and α-arabinosidase were probably identical to the higher molecular weight β-galactosidases, in agreement with Norden et al. (1974). The β-xylosidase activity was coincident with the β-glucosidase activity in both peaks, so it was possible that these were identical. It should be stressed, however, that the first peak was in the void volume where good resolution of large particles is not possible. Other evidence for the interrelationship of these glycosidase activities came from studies of the pH profiles of these enzymes in cultured skin fibroblasts. Once again it appeared that β-glucosidase and β-xylosidase were related and that the responses of β-fucosidase and α-arabinosidase were similar. The profile of β-galactosidase differed from that of β-fucosidase and α-arabinosidase and it was not easy to see the significance of this.

(b) STUDIES ON β-GLUCOSIDASE IN GAUCHER'S DISEASE.

RESULTS.

(i) Diagnosis of Gaucher's disease using cultured skin fibroblasts.

All the cases of Gaucher's disease studied here were first diagnosed using cultured skin fibroblasts with 4-methylumbelliferyl-β-D-glucopyranoside as substrate. As can be seen in Figure 74 some residual activity was present in Gaucher fibroblasts, but the activity in the three cases was well separated from that in the control. Also displayed is the β-glucosidase pH profile for fibroblasts from a patient diagnosed clinically as adult Gaucher's disease. In this case, the activity was at the bottom of, but not separated from the control range. The results of assays at pH 4.0 are displayed in Figure 75 (page 177 ). It can be seen that all

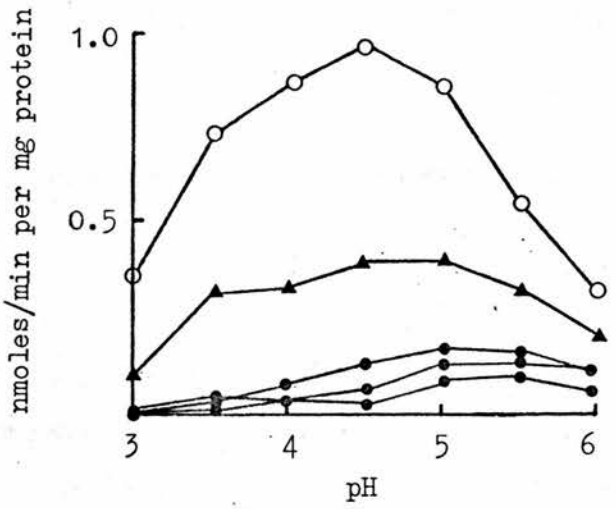


FIGURE 74 . β-glucosidase pH profiles of cultured skin fibroblasts from a typical control (o), cases of Gaucher's disease (•) and a patient diagnosed clinically as adult Gaucher's disease (▲).

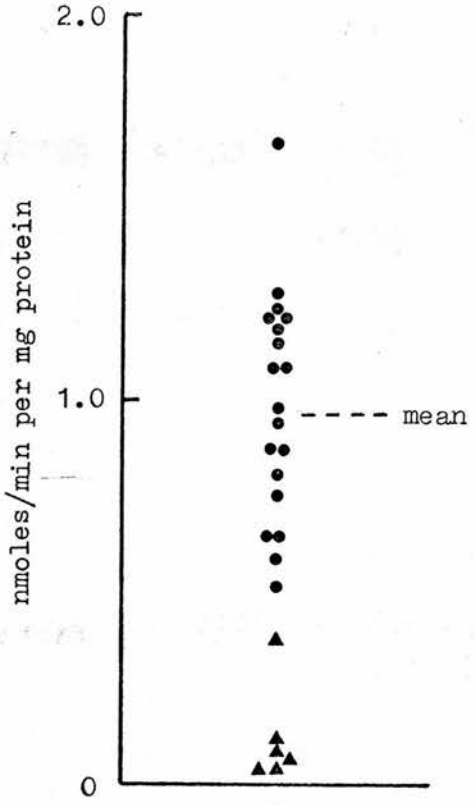


FIGURE 75 . Scattergram showing fibroblast β-glucosidase activity at pH 4.0 in controls (•) and Gaucher patients (▲).

the cases of Gaucher's disease were well separated from the controls, except the one case of adult Gaucher's disease. No further work was done on this case and no other cases of adult Gaucher's disease were encountered apart from cells obtained from the USA from a patient aged thirty five years with Gaucher's disease. The β-glucosidase activity in fibroblasts of this patient was in the range of the cases with infantile Gaucher's disease.

(ii) The β-glucosidase deficiency in Gaucher liver.

In the course of the study, two of the patients diagnosed using cultured skin fibroblasts died and tissues were obtained at autopsy. One patient (K.M.) was of the sub-acute neuronopathic type and died at four and a half years and the other (R.L.) was of the acute neuronopathic type and lived for only seven months. When the first of these (K.M.) died, the 4-methylumbelliferyl-β-glucosidase activity was determined in liver and, in Figure 76a (page 179 ), it can be seen that the activity in the Gaucher, although less than the control, was by no means deficient. The activity in the spleen of this case was much lower than the control, although even in this tissue, the residual activity was about 15% of the control level (Figure 76b, page 179 ).

It was probable that Gaucher liver was deficient in just one of the components of non-specific β-glucosidase. As two peaks of β-glucosidase activity had been obtained by Sephadex G-150 gel filtration of control human liver homogenates, this technique was used for Gaucher liver (R.L.). Once again, however, two peaks of activity were obtained, eluting in the same fractions as control liver β-glucosidases (Figure 77 , page 180 ). Peaks I and II were also obtained from the liver of the other case of Gaucher's disease (K.M.). The pH profiles for peaks I and II of both cases of Gaucher's disease and a control are shown in Figure 78 (page 181 ). There was little difference between Gaucher and control in peak II, but in peak I both Gaucher livers had markedly reduced activity at pH 4.0.

For each liver, the combined activity at pH 4.0 of the two

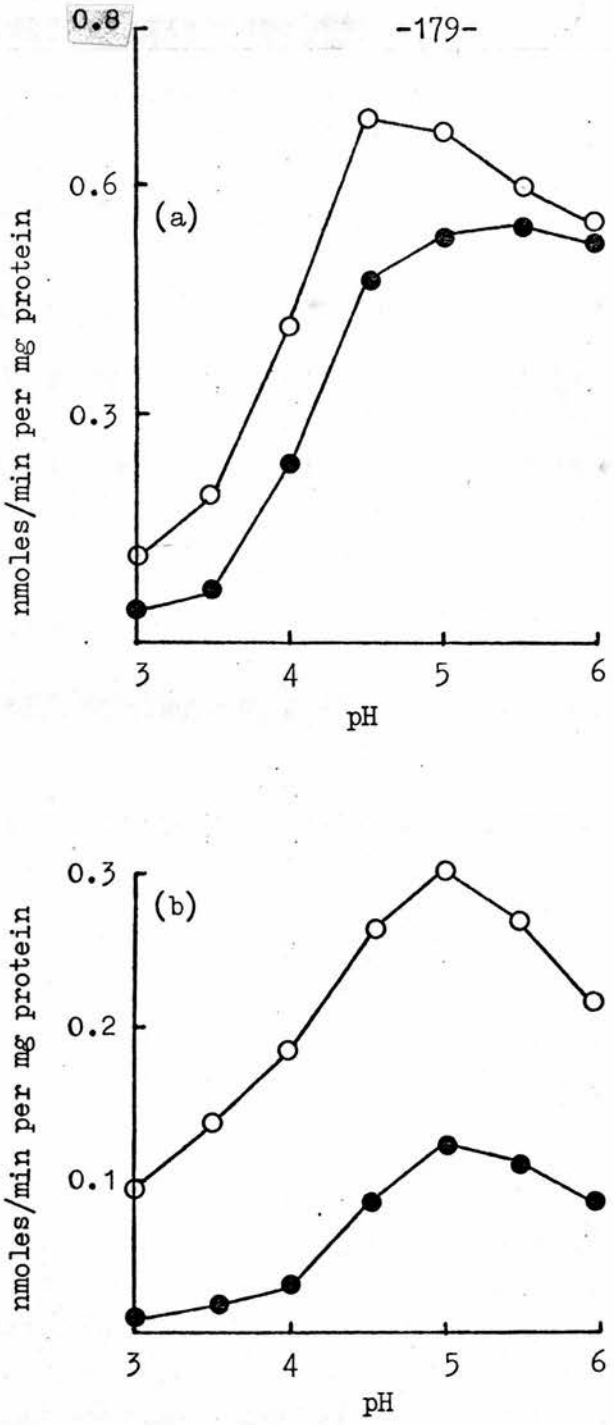


FIGURE 76 . β-glucosidase pH profile of (a) liver and (b) spleen of control (O) and Gaucher (K.M.) patient (●).

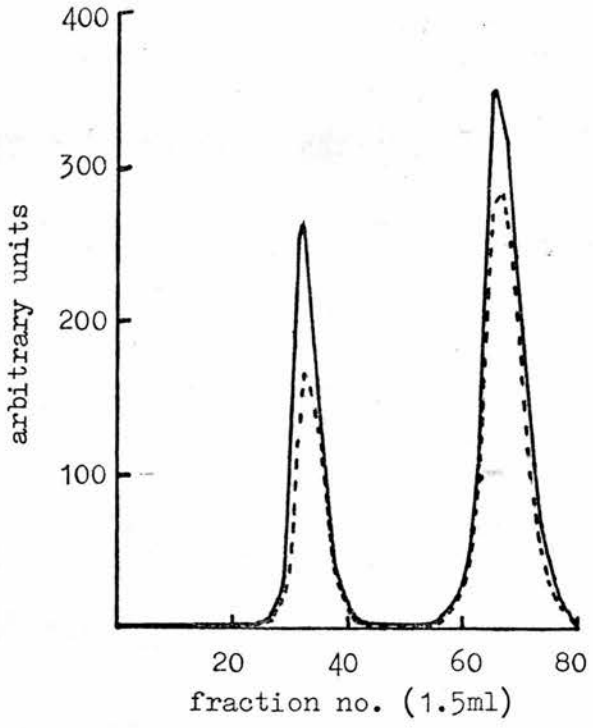


FIGURE 77 . Sephadex G-150 gel filtration of β-glucosidase from control (————) and Gaucher (R.L.) (- - - - -) liver.

separated Sephadex peaks was less than the activity of a water homogenate. This was particularly noticeable in the two Gaucher livers. It was suspected that this loss of activity was due to some component of the elution buffer, in the presence of which the enzyme had been assayed. When total homogenates of control and Gaucher liver were prepared in elution buffer, the latter was now shown to be deficient in β-glucosidase activity at pH 4.0 (Figure 79 , page 182 ).

The effects of EDTA and sodium chloride, major components of the elution buffer, on β-glucosidase activity were investigated. Extraction in 10mM-EDTA resulted in inhibition at pH 4.0 and

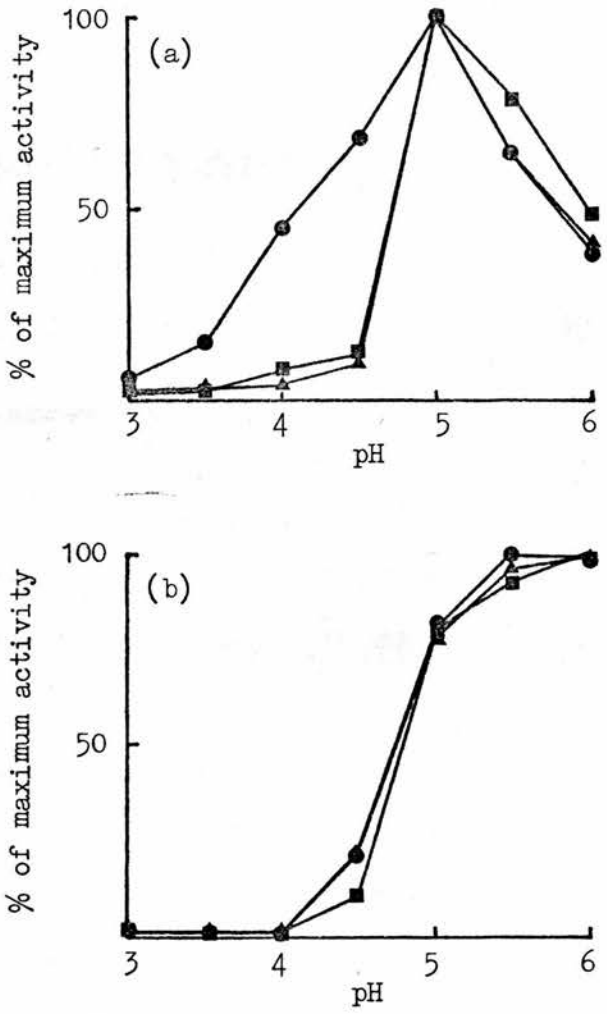


FIGURE 78 . β-glucosidase pH profiles of (a) peak I and (b) peak II from Sephadex G-150 gel filtration of control (●), Gaucher (R.L.) (▲) and Gaucher (K.M.) (■) liver.

activation at pH 5.5-6.0 for both control and Gaucher liver (Figure 80a, page 183 ). The effects of different concentrations of EDTA on activity at pH 4.0 is shown in Figure 81b (page 184 ). Extraction into 400mM-sodium chloride resulted in almost total inhibition of β-glucosidase activity at pH 4.0 for Gaucher liver and about 60% inhibition of control liver activity (Figure 80b,

page 183). The effect of different concentrations of sodium chloride on enzyme activity at pH 4.0 was investigated (Figure 81a , page 184 ) and extraction into 200mM-sodium chloride was optimal for distinguishing between Gaucher and control liver. When peaks I and II were dialysed against distilled water, the activity at pH 4.0-4.5 was raised markedly (Figure 82 , page 185).

Subsequently, it was shown that all the non-specific β-glucosidases present in Gaucher liver could be removed by preincubation at pH 4.0 in the presence of 50mM-sodium chloride (Figure 83 , page 186). After this treatment, the activity was not restored when the homogenate was assayed at more neutral pH values. It appeared, therefore, that inhibition at pH 4.0 was a result of irreversible inactivation. If sodium chloride was removed first by dialysis, inactivation did not occur to the same extent.

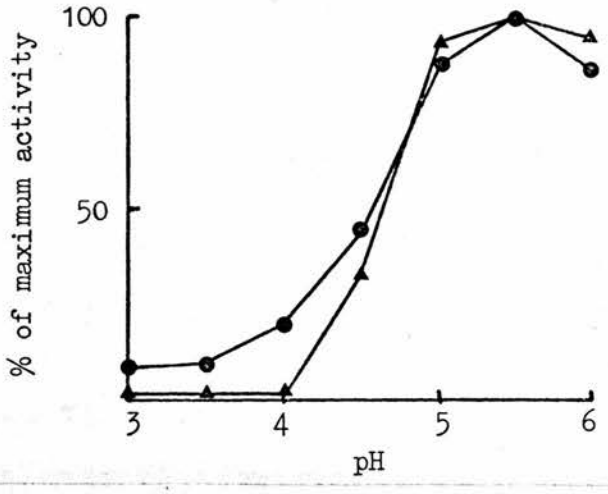


FIGURE 79 . β-glucosidase pH profiles of control (●) and Gaucher (R.L.) (▲) liver, extracted into 10mM-sodium phosphate buffer, pH 6.0, containing 10mM-EDTA and 400mM-NaCl.

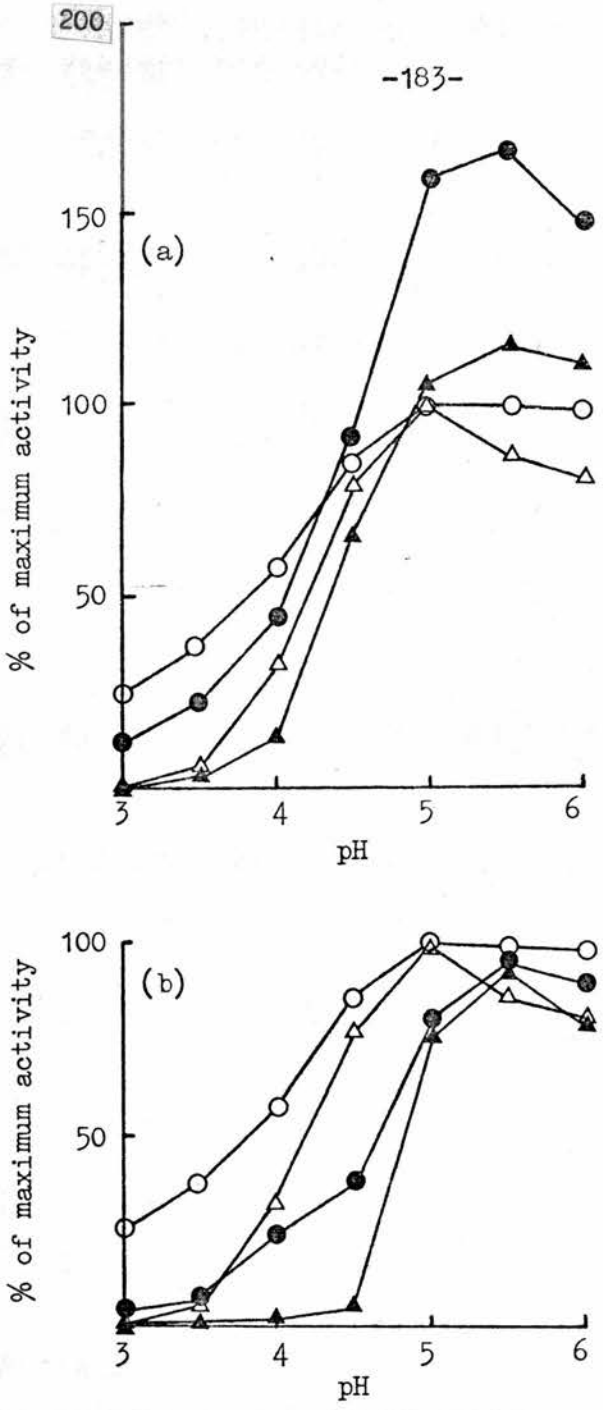


FIGURE 80. Effect of extraction into (a) 10mM-EDTA and (b) 400mM-NaCl on the β-glucosidase pH profile of control (○, ●) and Gaucher (R.L.) (△, ▲) liver. Open symbols indicate no addition and closed symbols, the addition of EDTA or NaCl.

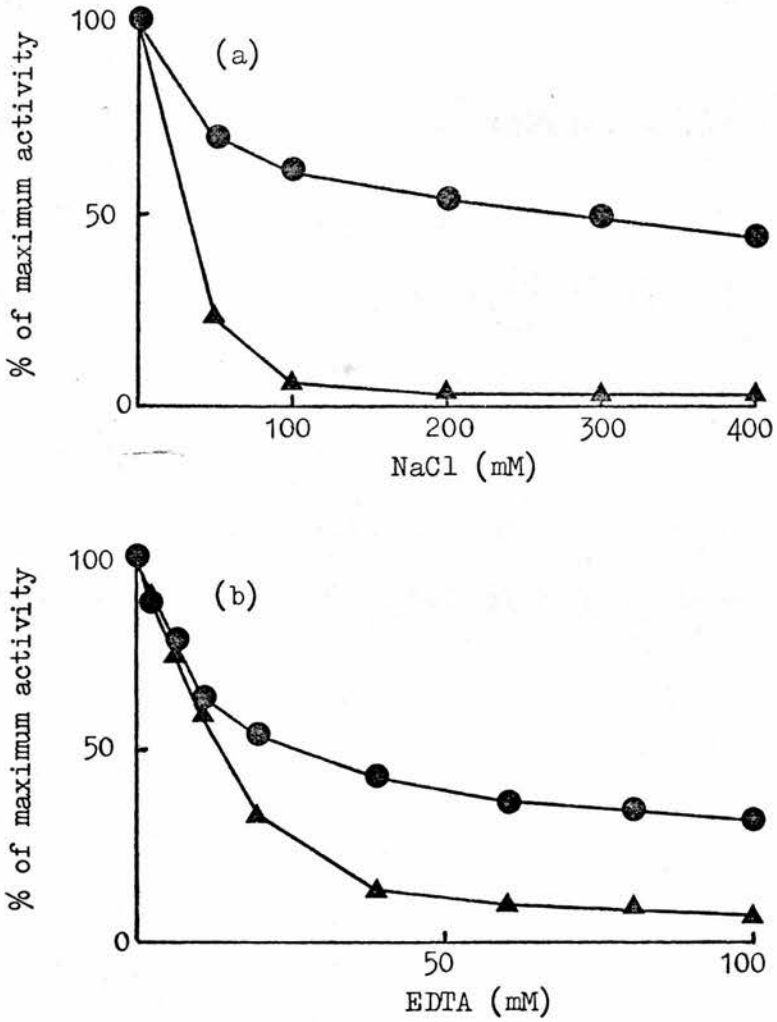


FIGURE 81 . Effect of the concentration of (a) NaCl and (b) EDTA in the extract of control (●) and Gaucher (R.L.) (▲) liver on β-glucosidase activity at pH 4.0.

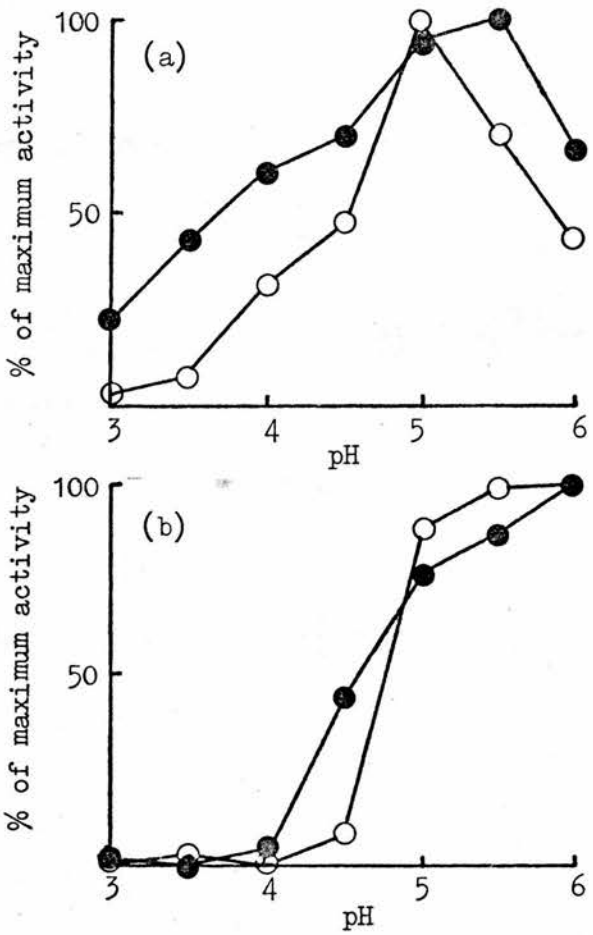


FIGURE 82. Reversal of the effect of Sephadex G-150 elution buffer on (a) peak I and (b) peak II of control liver β-glucosidase by dialysis against distilled water.

(○) undialysed (●) dialysed

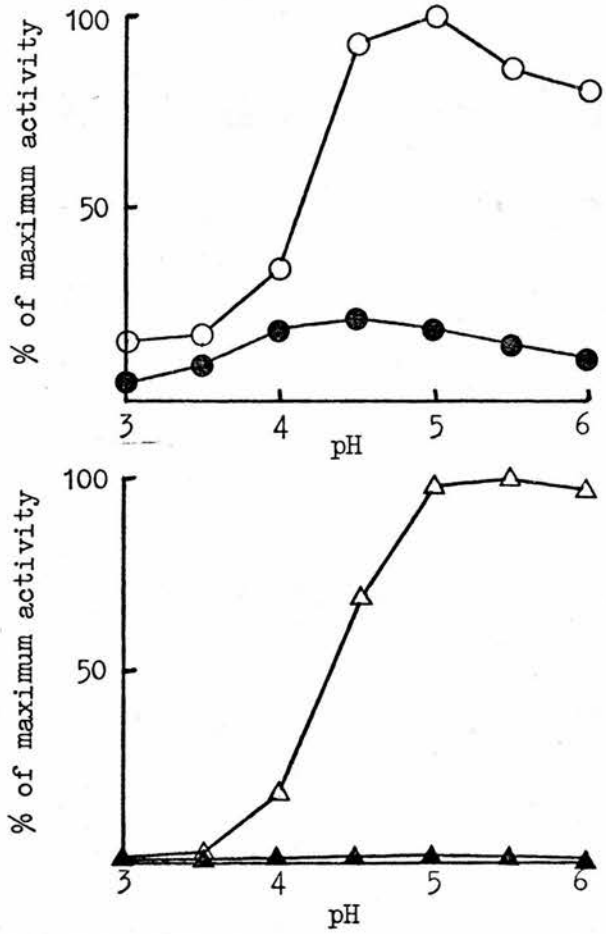


FIGURE 83. Effect of preincubation in 50mM-NaCl at pH 4.0 on control (○, ●) and Gaucher (R.L.) (△, ▲) liver. Open symbols indicate untreated and closed symbols, treated samples.

(iii)The activity of other glycosidases in Gaucher's disease.

The common identity of certain components of the glycosidases, β-galactosidase, β-glucosidase, β-fucosidase, β-xylosidase and α-arabinosidase was investigated in controls and cases of $G_{M1}$ gangliosidosis and Gaucher's disease. Gaucher liver had a similar elution profile to controls for all enzymes assayed (Figure 84 , page 188 ). As had been observed earlier (Figure 70 , page 166 ), the elution profiles of β-galactosidase, β-fucosidase and α-arabinosidase were very similar, apart from their relative activities in the final peak. β-Glucosidase and β-xylosidase each had two peaks, coincident with each other and the first and last peaks of the other three activities assayed.

The elution profile for three of these enzyme activities was quite different when a liver homogenate from a case of $G_{M1}$ gangliosidosis was eluted from the Sephadex column (Figure 85 , page 189 ). As expected, the non-specific β-galactosidase activity was very low in this liver, the major peak corresponding to the final peak of control and Gaucher liver. A small fraction of the activity was eluted with the void volume, but this was less than 1% of the control level for peak I. Similarly, the activities of β-fucosidase and α-arabinosidase were also deficient in this liver apart from the final peak. β-Glucosidase and β-xylosidase peaks were as for control and Gaucher livers.

The peak tubes of peaks I and II β-glucosidase were taken for pH profiles of all five glycosidases. Samples of the β-galactosidase activity eluted between the first and last peaks were taken for pH profiles of β-galactosidase, β-fucosidase and α-arab-

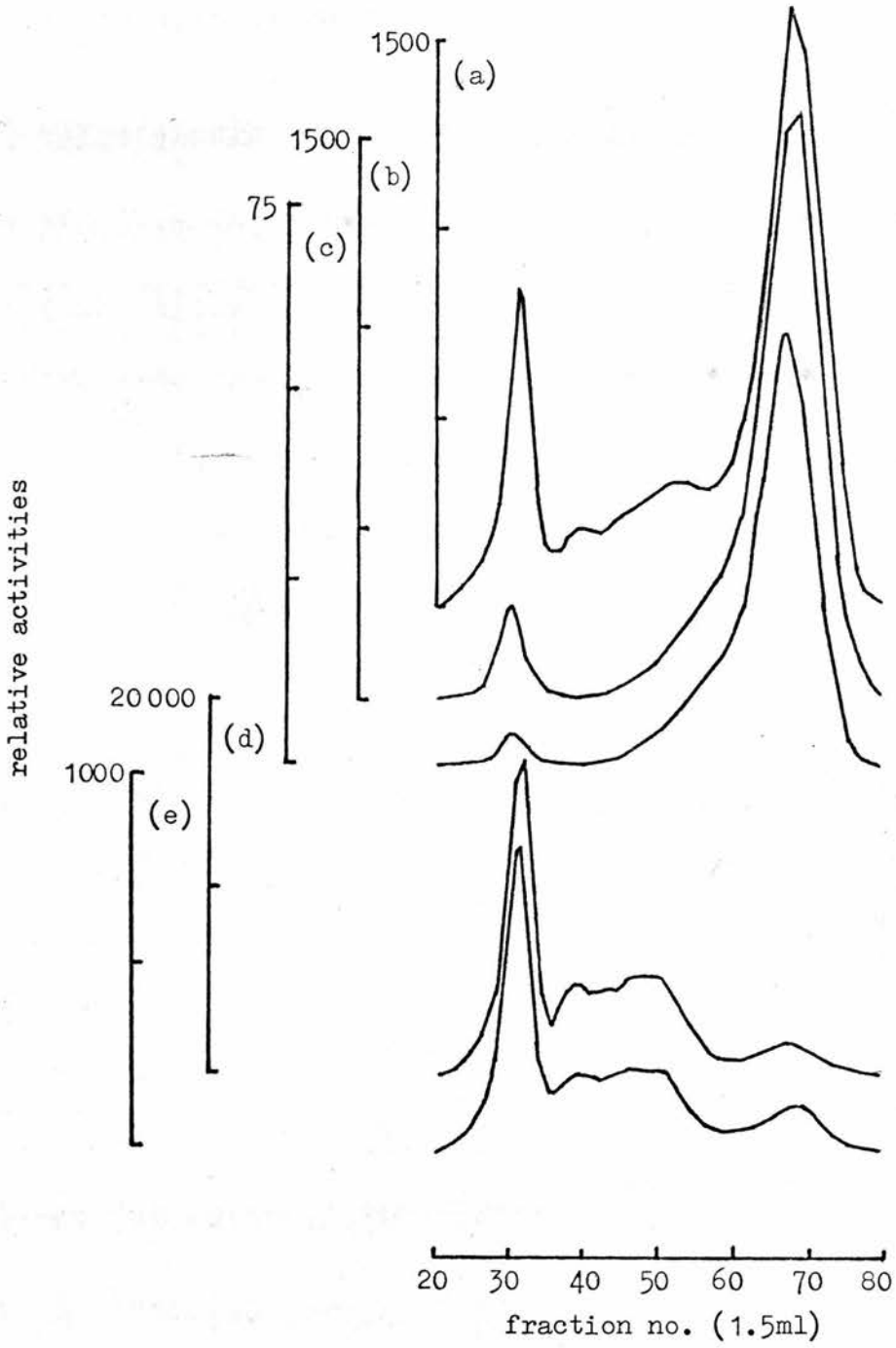


FIGURE 84. Sephadex G-150 gel filtration of (a) β-D-fucosidase, (b) β-D-glucosidase, (c) β-D-xylosidase, (d) β-D-galactosidase and (e) α-L-arabinosidase of Gaucher liver.

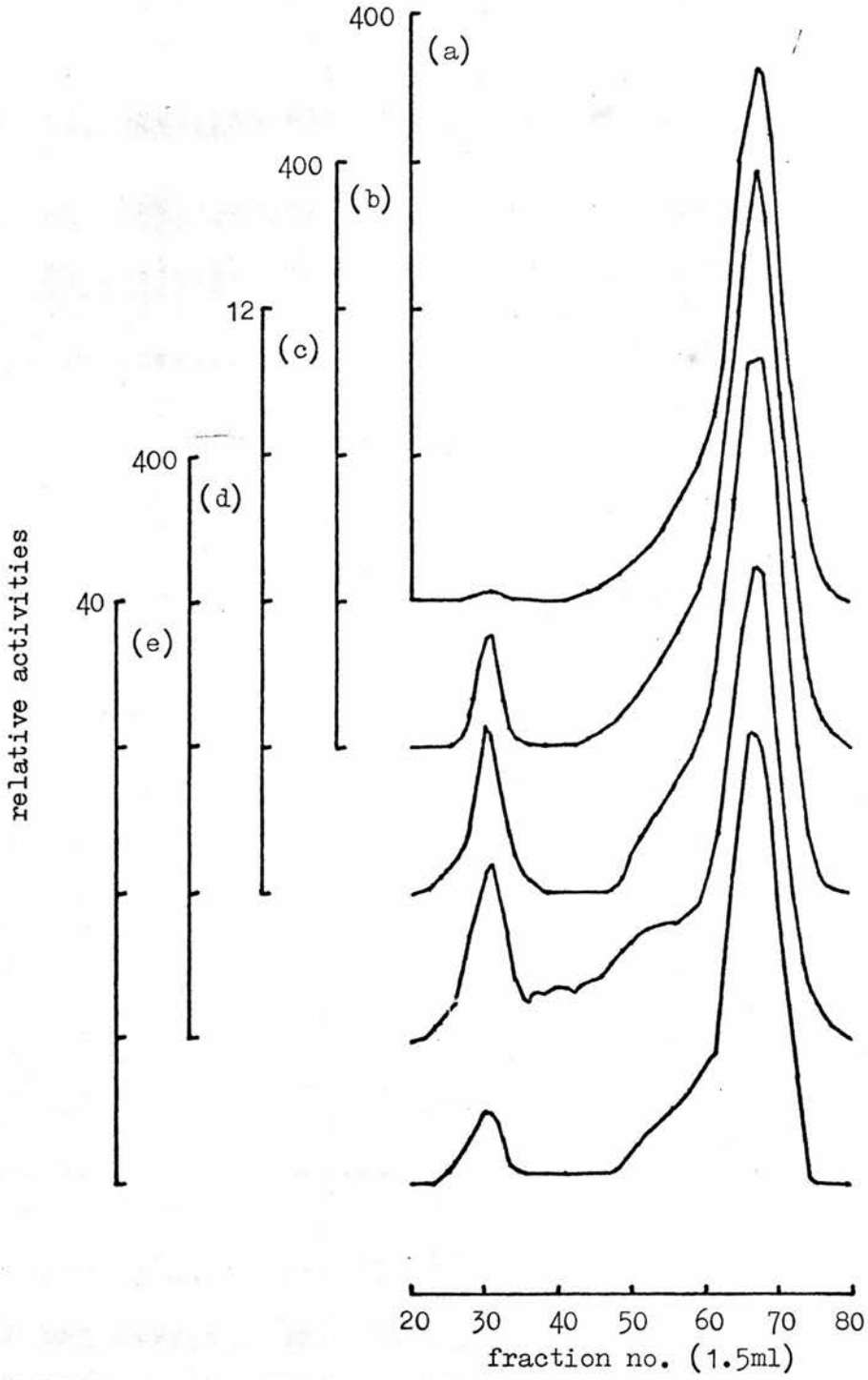


FIGURE 85. Sephadex G-150 gel filtration of (a) β-D-fucosidase, (b) β-D-glucosidase, (c) β-D-xylosidase, (d) β-D-galactosidase and (e) α-L-arabinosidase of $G_{M1}$ gangliosidosis liver.

inosidase for Gaucher and control. There was insufficient of this intermediate enzyme activity in the case of $G_{M1}$ gangliosidosis to merit assay. The results for all these profiles are displayed in Figure 86 (page 191 ). It was observed that β-galactosidase peak I differed from peaks II and III in pH optimum and activity at pH 3.0-3.5. This was also found for α-arabinosidase and β-fucosidase, although the profile of β-fucosidase peak III was probably somewhat affected by contamination from the very active peak IV. The pH profile of peak IV was similar for all the glycosidases assayed, allowing for some contamination by peak III where present.

The pH profiles for four of the glycosidases in Gaucher and control fibroblasts are shown in Figure 87 (page 192 ). Gaucher fibroblasts were deficient in β-xylosidase but none of the other glycosidases differed from the control. $G_{M1}$ gangliosidosis fibroblasts had normal β-glucosidase and β-xylosidase activities, but were deficient in β-fucosidase and α-arabinosidase as well as β-galactosidase.

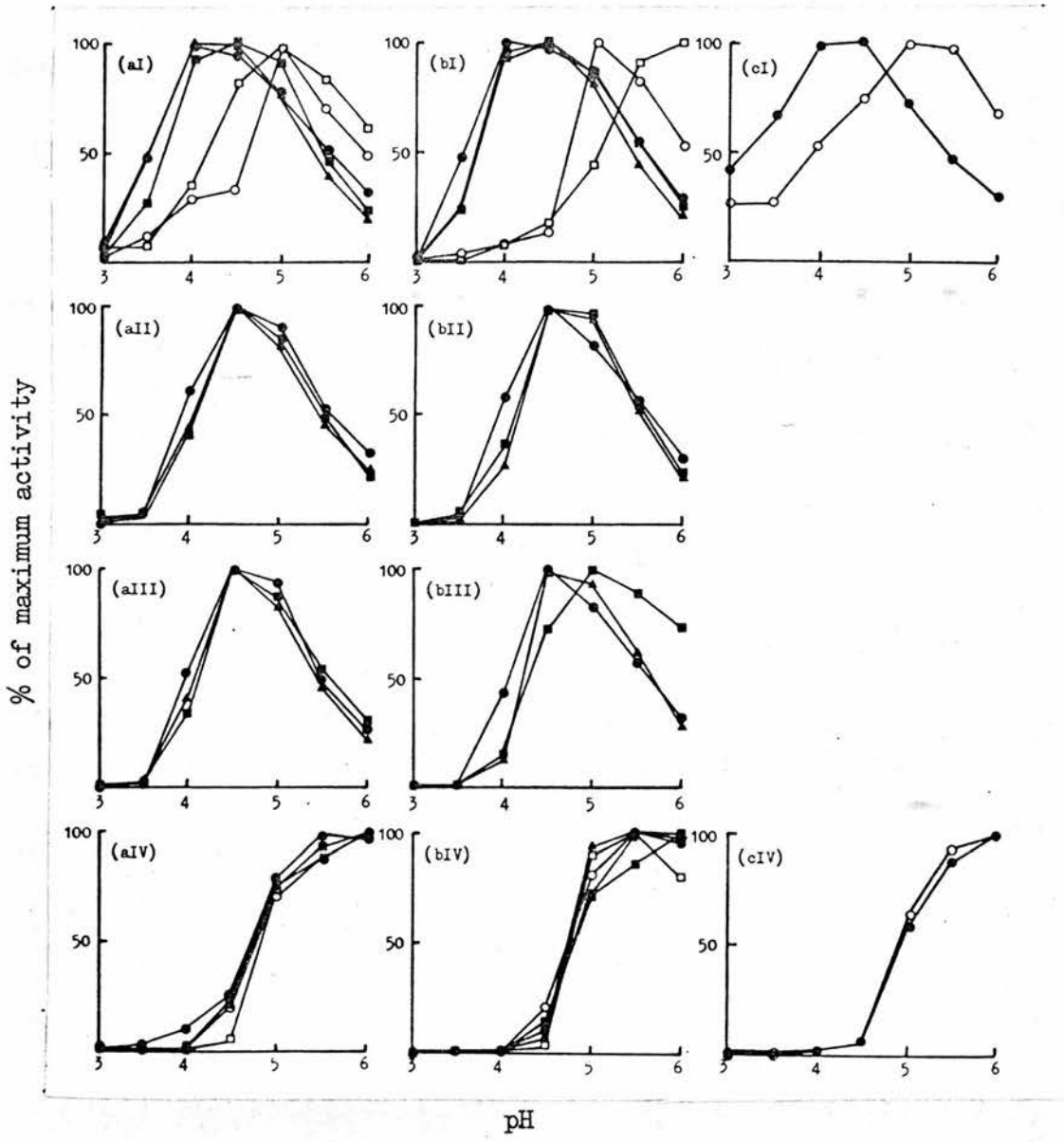


FIGURE 86. pH profiles of β-glucosidase (○), β-xylosidase (□), β-galactosidase (●), β-fucosidase (■) and α-arabinosidase (▲) of peaks I-IV from Sephadex G-150 gel filtration of (a) control, (b) Gaucher and (c) $G_{M1}$ Gangliosidosis liver.

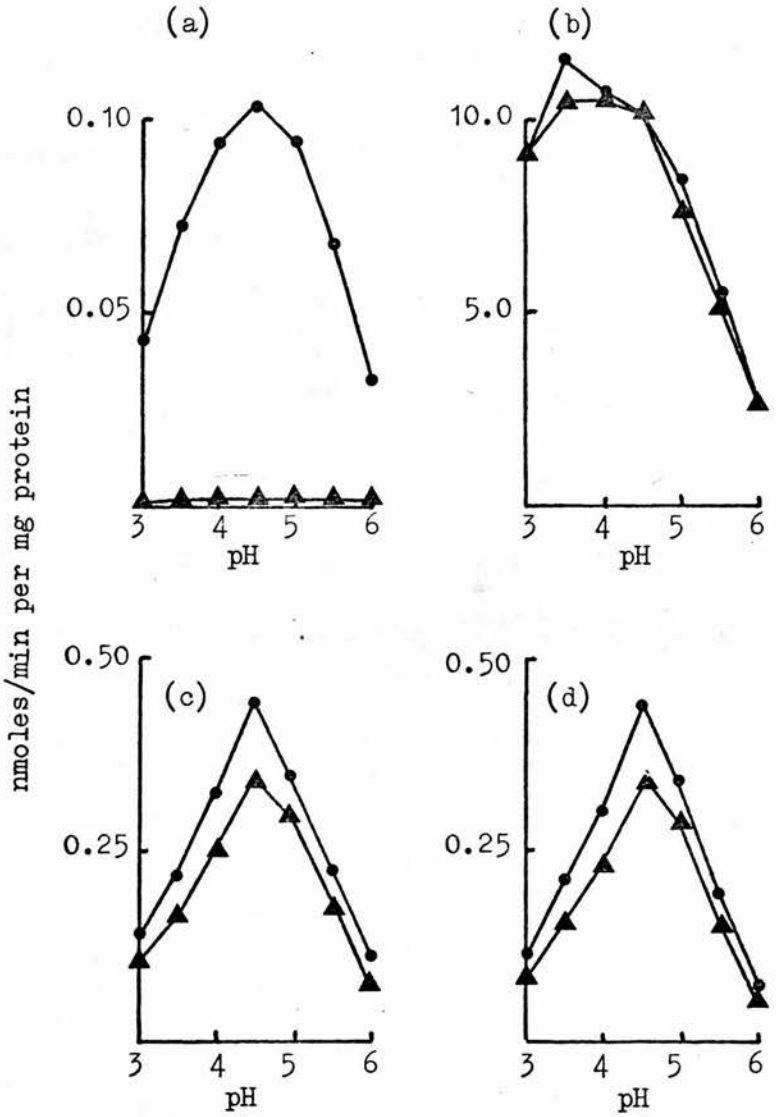


FIGURE 87 . pH profiles of (a) β-D-xylosidase, (b) β-D-galactosidase, (c) β-D-fucosidase and (d) α-L-arabinosidase for control (•) and Gaucher (▲) fibroblasts.

(iv) Factors affecting the pH profile of Gaucher fibroblasts.

The effects of different conditions of assay on the pH profile of β-glucosidase, demonstrated in control cells, were studied in fibroblasts from patients with Gaucher's disease. Gaucher fibroblast extracts were assayed in the presence of Triton X-100 (0.02%$^{V}$/v), sodium taurocholate (0.1%$^{W}$/v) or both. There was no activation at any pH and indeed, for all three combinations, inhibition occurred (Figure 88 ). Under control conditions, the optimum was pH 5.0-5.5 and only in the presence of Triton X-100 was any change characteristic of control cells (Figure 63 , page 158 ) observed.

The effect of phosphatidylserine on the residual β-glucosidase activity of Gaucher fibroblasts (Figure 89 , page 194 ) was similar, but not as marked as its effect had been on controls (Figure 65 , page 160 ).

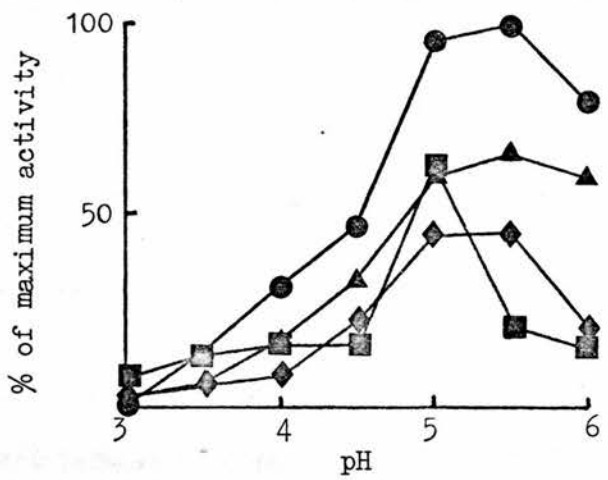


FIGURE 88 . Effect of Triton X-100 (0.02%$^{V}$/v) (■), sodium taurocholate (0.1%$^{W}$/v) (▲) and Triton X-100 (0.02%$^{V}$/v) with sodium taurocholate (0.1%$^{W}$/v) (◆) compared with no addition (●) on the residual β-glucosidase activity of Gaucher fibroblasts.

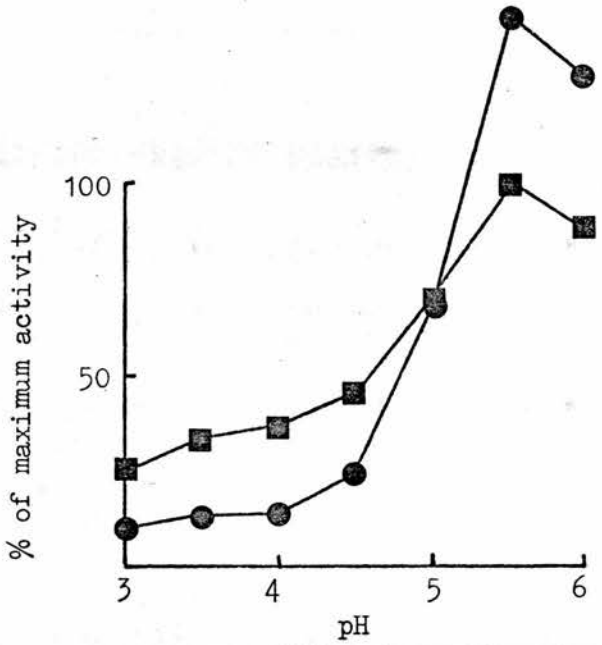


FIGURE 89. Effect of phosphatidylserine on the residual β-glucosidase activity of Gaucher fibroblasts.

(●) 0.15mM phosphatidylserine (■) no addition

Saline suspension had similar effects on the β-glucosidase activity of Gaucher fibroblasts (Figure 90 , page 195 ) as had been found for controls (Figure 66 , page 162 ). The sharpening of the pH optimum, however, was not as marked as in controls and the residual activity at pH 5.5, although inhibited, was still sometimes greater than the activity at pH 4.0.

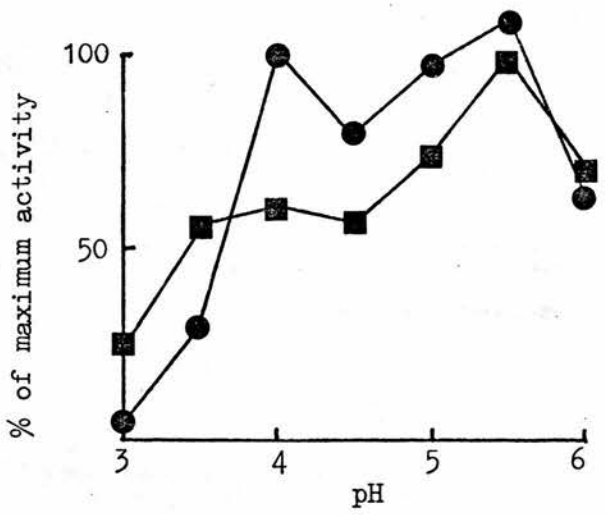


FIGURE 90. Comparison of the effects of saline suspension (●) and water lysis (■) on the β-glucosidase pH profile of Gaucher fibroblasts.

DISCUSSION.

(i) The β-glucosidase activity of Gaucher liver.

It has been reported (Beutler et al., 1971; Ho et al., 1972) that cultured skin fibroblasts from patients with adult Gaucher's were deficient in 4-methylumbelliferyl-β-glucosidase activity. In this study, cases of neuronopathic Gaucher's disease were also found to be deficient, but in one of the two cases of adult Gaucher's disease investigated only a partial deficiency was noted.

Liver from two patients with neuronopathic Gaucher's disease was obtained at autopsy and the 4-methylumbelliferyl-β-glucosidase activity was not deficient. Similar findings were reported by Patrick (1965), who found that the activity of p-nitrophenyl-β-glucosidase in Gaucher liver was twice that of controls. However, the activity of non-specific β-glucosidase in Gaucher spleen was much reduced and the results presented here are in agreement with this finding.

Ockerman (1968) claimed that Gaucher liver was deficient in low molecular weight β-glucosidase, the only peak of activity he obtained by gel filtration of control liver. However, if low speed centrifugation was used, it was possible to separate the β-glucosidase of control liver into two peaks by Sephadex G-150 gel filtration. It would appear, therefore, that Ockerman was only looking at a part of the total β-glucosidase activity. When Gaucher liver was investigated, however, it was found that neither peak of β-glucosidase was deficient when assayed at pH 5.0.

(ii) Identification of the deficiency in Gaucher liver.

When the two peaks from the Sephadex column were taken for pH profiles, it was shown that Gaucher liver differed from control liver in peak I only as expected from the reported position of glucosyl ceramide β-glucosidase (Ho, 1973). The deficiency in peak II reported by Ockerman (1968) was more difficult to explain, but as controls, apparently deficient in peak II, have been observed (Butterworth et al., 1972), it is possible he was unlucky in his choice of cases of Gaucher's disease. Another possibility, however, is that peaks I and II are genetically linked as with hexosaminidases A and B and that some cases of Gaucher's disease are deficient in both peaks of β-glucosidase.

(iii) Use of sodium chloride in the diagnosis of Gaucher's disease in liver.

The ease of diagnosis using the separated peaks suggested that the elution buffer was suppressing interfering β-glucosidase components. This was indeed shown to be the case and the activity at pH 4.0 was reduced in the presence of either 400mM-sodium chloride or 10mM-EDTA. There was also activation at pH 4.5-6.0 in the presence of EDTA. A similar effect of sodium chloride on soluble β-glucosidase and its associated β-galactosidase activity had been reported previously (Ho and O'Brien, 1971b). Although the activity could be regenerated by dialysis to remove sodium chloride, the components of non-specific β-glucosidase which normally mask the deficiency in Gaucher liver were permanently inactivated by preincubation of homogenates in 50mM-sodium chloride at pH 4.0.

(iv) Activities of other glycosidases said to have common identity with β-glucosidase in Gaucher tissues.

Studies on the five glycosidases suggested that β-glucosidase and β-xylosidase activities were attributable to the same enzyme molecule at least insofar as the enzyme deficiency in Gaucher's disease was concerned. Similar conclusions were reached by Öckerman (1968) and Chester et al. (1976). These findings were also in agreement with those of Öckerman (1968) who claimed that the final peak of β-galactosidase on Sephadex G-150 also had β-glucosidase and β-xylosidase activity. This peak was also shown to have β-fucosidase and α-arabinosidase activity. Purified β-galactosidase has previously been shown to have β-fucosidase and α-arabinosidase activities (Norden et al., 1974). The elution profile from Sephadex G-150 was very similar for β-galactosidase, β-fucosidase and α-arabinosidase, and in $G_{M1}$ gangliosidosis liver all three activities were absent apart from a little activity in peak I and the final peak which was unaffected. Gaucher liver was not deficient in β-fucosidase as reported by Chester et al. (1976) nor were Gaucher fibroblasts deficient in α-arabinosidase as reported by Hultberg et al. (1973).

(v) The residual β-glucosidase of Gaucher fibroblasts.

The nature of the residual β-glucosidase in Gaucher fibroblasts was of some interest. It has already been shown that, as in leucocytes, the β-glucosidase activity of control fibroblasts is found only in the void volume of Sephadex G-150. This could have been due to an inability to detect a low activity of soluble β-glucosidase (peak IV), but as peak IV β-galactosidase was present in $G_{M1}$ gangliosidosis liver and no activity was detectable at pH 5.5-6.0 in fibroblasts from this case, the possibility is unlikely. It was assumed, therefore, that the residual β-glucosidase in Gaucher fibroblasts would have been eluted in peak I. As the activity was so low, no attempt was made to establish that this was indeed the case.

The effect of assay conditions yielded some information as to the nature of the residual β-glucosidase. In both Gaucher and control fibroblasts, phosphatidylserine inhibited β-glucosidase activity at acid pH and activated at more neutral pH. Although this was not as marked in the case of Gaucher's disease as in the control, it is reasonable to suggest that the enzyme was the same in both. The effects of sodium taurocholate and Triton X-100 were less helpful as the optimum for β-glucosidase in Gaucher fibroblasts was not dissimilar to that imposed on control fibroblast β-glucosidase by these reagents. There was some evidence that saline suspension affected the pH profile of the residual β-glucosidase of Gaucher fibroblasts, but once again, the pH optimum, biased as it was to a more neutral pH, masked the change. The response of the pH profile to different conditions suggested

that β-glucosidase might exist in more than one interconvertible forms. The residual β-glucosidase in Gaucher fibroblasts might be one of these forms, with some activity towards artificial substrates, but little towards glucosyl ceramide. The deficiency in Gaucher's disease could be due to an inability to convert this form to active enzyme. It was interesting that the pH profile of the residual β-glucosidase in Gaucher fibroblasts was very similar to that reported for factor C in the absence of factor P (Ho and O'Brien, 1971a). However, this residual activity may be genetically unrelated to the glucocerebrosidase activity, in the same way as it has been established that hexosaminidase C which remains in Sandhoff's disease is unrelated to hexosaminidases A and B.

# GENERAL DISCUSSION

GENERAL DISCUSSION.

(i) The use of artificial substrates for the detection of hydrolase deficiencies in lysosomal storage disorders.

Generally, patients with lysosomal storage disorders have been found to lack the catalytic activity of a specific degradative enzyme. Often these enzymes exist in multiple forms that may or may not be genetically linked. Using artificial substrates, it is more likely that the multiple forms will be unrelated than when a natural substrate is used. Nevertheless, as in the case of hexosaminidases A and B, components may be genetically related but have different natural substrates (Sandhoff et al., 1977). In this thesis, three hydrolases have been studied and in each case neutral as well as acid hydrolase activities were detected. It is possible that some neutral hydrolases may be transferases in vivo and it may be significant that neutral α-mannosidase is activated by cobalt (Phillips et al., 1974), which is also an activator of galactosyl transferase activity (Butterworth, 1974). The results suggested that neutral and acid activities were unrelated and methods were developed for the removal of activities which hinder the diagnosis of storage disorders using low specificity artificial substrates.

(ii) Interrelationships of different enzyme components.

Although patients with Tay-Sachs disease were found (Svennerholm, 1962) to store $G_{M2}$ ganglioside, a lipid which could be degraded using calf brain N-acetyl-β-D-hexosaminidase (Frohwein and Gatt, 1967), they were found not to have a total deficiency of N-acetyl-β-D-hexosaminidase activity (Sandhoff et al., 1968). It was eventually found (Okada and O'Brien, 1969), however, that Tay-Sachs patients were deficient in one particular hexosaminidase component, namely Hex-A. The panethnic form of $G_{M2}$ gangliosidosis, Sandhoff's disease, which accounts for one third of cases, was recognised as a distinct variant with a total deficiency of N-acetyl-β-D-hexosaminidase by Sandhoff et al. (1968). It is possible that other diseases may be due to a deficiency of one component only. Indeed, it would seem that patients with Niemann-Pick disease type C may be deficient in certain but not all components of sphingomyelinase (Callahan et al., 1974; 1975; Besley, 1977).

It has been claimed that infantile Pompe's disease has reduced neutral maltase activity in heart and muscle (Angelini and Engel, 1972) suggesting that the acid and neutral components are under common regulatory control (Angelini and Engel, 1973), but, in work presented in this thesis (page 112), neutral α-glucosidase was not significantly reduced in Pompe fibroblasts. It is possible, however, that stored glycogen may inhibit neutral α-glucosidase activity similarly to the inhibition of β-galactosidase by glycosaminoglycans in Hunter and Hurler syndromes (Kint, 1973; 1974).

Similarly, although the glucocerebrosidase activity, shown

to be deficient in Gaucher's disease (Brady et al., 1965; Patrick, 1965) is associated with the particulate non-specific β-glucosidase activity (Ho, 1973), Gaucher patients have been reported (Öckerman, 1968; Kanfer et al., 1975) who were also deficient in soluble β-glucosidase activity. It has been suggested (Peters et al., 1975) that the particulate glucocerebrosidase and soluble non-specific β-glucosidase are genetically related. Neither of the cases encountered in this study, one infantile and one juvenile, was deficient in low molecular weight (soluble) β-glucosidase in liver (page 180). Control livers have, however, been shown to lack soluble β-glucosidase activity (Butterworth et al., 1972; Cheetham et al., 1978) and it is possible that a deficiency of this enzyme has no clinical significance.

(iii)The interrelationships of hexosaminidase components.

It was clear from the different storage materials (Sandhoff et al., 1971) in Tay-Sachs and Sandhoff's diseases, that hexosaminidases A and B had different substrate specificities and it would appear that only Hex-A can attack the sialated form of stored lipid, $G_{M2}$ ganglioside (Sandhoff et al., 1977). As hexosaminidases B, $I_1$ and $I_2$ are all present in Tay-Sachs tissues (Young et al., 1970), these components are probably closely related. Similarly, the serum and tissue forms of Hex-A are both deficient in Tay-Sachs disease (Okada and O'Brien, 1969) suggesting that these too are closely linked.

In I-cell disease, the activity of certain acid hydrolases is raised in serum (Wiesmann et al., 1971) and in culture medium in which the I-cell fibroblasts have been grown (Wiesmann and Herschkowitz, 1974); the activity of these enzymes in fibroblast extracts, however, is decreased (Leroy et al., 1972). It was proposed (Hickman and Neufeld, 1972) that in I-cell disease the recognition site enabling certain hydrolases to be taken up into lysosomes is altered. It was noted, however, that I-cell fibroblasts released only serum forms of hydrolases into the medium, whereas control cells had both serum and tissue forms in the surrounding medium (Ellis, Willcox and Patrick, 1975), suggesting that the deficiency was in the biosynthetic pathway of tissue forms. It is likely that, if tissue and extracellular forms are derived from the same biosynthetic pathway, the activity of extracellular forms will be raised in I-cell disease. This is so and as serum and tissue forms of Hex-A are deficient in Tay-Sachs disease, whereas

components B, $I_1$ and $I_2$ are retained, it is likely that tissue Hex-A and serum Hex-A have a common precursor as have hexosaminidases B, $I_1$ and $I_2$.

It would appear that Hex-S contains the unique subunit of tissue Hex-A (Beutler and Kuhl, 1975). Another component, Hex-S', was reported by Beutler, Kuhl et al. (1975) to elute from DEAE-cellulose before Hex-S. In this study, however, the component corresponding to Hex-S' had a lower isoelectric point than Hex-S (pages 67, 78). The relationship of Hex-S' to Hex-S closely paralleled that of serum Hex-A to tissue Hex-A using these two techniques (Ikonne and Ellis, 1973; this thesis pages 31, 32). It would seem likely, therefore, that just as Hex-S provides the unique subunit differentiating Hex-A from Hex-B, Hex-S' is the source of the unique subunit of serum Hex-A. Similarly, there may be two forms of Hex-B subunit, formed from a common precursor which may explain the relationship of Hex-B to components $I_1$ and $I_2$. Hex-B may contain all tissue subunits, Hex-$I_1$ half tissue and half serum subunits, and Hex-$I_2$ all serum subunits. If there were tissue and serum forms of $\alpha$ and $\beta$ subunits, aggregating as half molecules, four forms of Hex-A would be predicted, unless it were postulated that serum and tissue forms of $\alpha$ subunit could only form serum Hex-A and tissue Hex-A respectively with one form of $\beta$ subunit. It is possible, however, that more than two forms of Hex-A do exist as demonstrated by sucrose gradient isoelectric focusing of leucocytes (page 34) and the multiple bands of hexosaminidase obtained by gel isoelectric focusing of serum (Hayase and Kritchevsky, 1973). The proposal of Srivastava et al. (1976) that

hexosaminidases B, $I_1$, $I_2$, A, S' and S are all hexamers and constituted $\beta_6$, $\beta_5\alpha_1$, $\beta_4\alpha_2$, $\beta_3\alpha_3$, $\beta_2\alpha_4$ and $\beta_1\alpha_5$ respectively does not allow the presence of hexosaminidases $I_1$ and $I_2$ in Tay-Sachs disease (Young et al., 1970) nor the residual Hex-S activity in Sandhoff's disease (Beutler, Kuhl et al., 1975; Ikonne et al., 1975). Other workers (Geiger and Arnon, 1976; Lee and Yoshida, 1976; Beutler et al., 1976) claim that hexosaminidases A and B are tetramers, but Srivastava (Wiktorowicz et al., 1977) has recently reiterated his contention that the hexosaminidase components are hexamers. It is proposed in this thesis that the various components of hexosaminidase are combinations of tissue $\alpha$, $\beta$ and serum $\alpha'$, $\beta'$ subunits as summarised below. Components I-V are as described on pages 33 and 34.

| designation | proposed constitution | approximate pI |
|---|---|---|
| S' | $\alpha'_{2n}$ | 4.15 |
| S(I) | $\alpha_{2n}$ | 4.40 |
| serum A(II) | $\alpha'_n\beta'_n$ | 4.75 |
| III | $\alpha'_n\beta_n$ | 4.90 |
| tissue A(IV) | $\alpha_n\beta_n$ | 5.25 |
| V | $\alpha_n\beta'_n$ | 5.40 |
| $I_2$(P) | $\beta'_{2n}$ | 6.50 |
| $I_1$ | $\beta_n\beta'_n$ | 7.20 |
| B | $\beta_{2n}$ | 8.00 |

Hex-C was present in both Tay-Sachs and Sandhoff's disease (page 65), as also found by Penton et al. (1975). This, as well as its more neutral pH optimum (page 39) and greater affinity for

the glucosaminidase substrate (page 40), suggested that it was not related to the other hexosaminidases and is, therefore, not discussed here.

(iv) The α-glucosidase components.

Four major α-glucosidase components were encountered in this study. Acid and neutral components were found in liver, cultured cells and lymphocytes (page 94), but the latter of the two components was not extensively studied as it had little activity at pH 4.0 and so did not interfere with the diagnosis of Pompe's disease (page 112) as had been feared (Fujimoto et al., 1976). Two other components, one found in kidney and leucocytes (pages 94, 113) and the other in amniotic fluid (page 94), which have neutral pH optima but considerable activity at pH 4.0 are designated intermediate components to differentiate them from the other two forms. It was confirmed (page 113) that the intermediate component was retained in kidney (Steinitz and Rutenberg, 1967) and leucocytes (Koster et al., 1972) of patients with Pompe's disease. Using inhibitors, it was established that the intermediate components of kidney and leucocytes were similar (pages 125-131), but differed from that of amniotic fluid (page 133). It had previously been shown that kidney and amniotic fluid had different maltase components (Salafsky and Nadler, 1971), but other reports (Koster et al., 1974; Koster et al., 1976) using glycogen as substrate suggest that kidney and leucocyte components also differ. Amniotic fluid α-glucosidase may be an altered form of the kidney enzyme with a different response to inhibitors.

Kidney α-glucosidase has a greater affinity for maltose than the acid α-glucosidase of liver (page 122), but its 4-methylumbelliferyl-α-D-glucosidase activity was inhibited non-competitively (phosphate-citrate buffers) by maltose (page 120). It would seem,

therefore, that this enzyme has two active sites with affinity for maltose, consistent with an in vivo transglucosylase function as proposed by de Burlet and Sudaka (1977). That this enzyme is found in kidney but not in liver suggests that it is possibly involved in transport mechanisms. Also of interest is the presence of this enzyme in polymorphonuclear cells, but absence from lymphocytes (page 133), indicating that the latter are more suitable for the diagnosis of Pompe's disease.

Only one hydrolytic site for maltose and 4-methylumbelliferyl-α-D-glucopyranoside was detected for liver acid α-glucosidase (pages 120,121) in agreement with de Barsy et al. (1972), but not with Koster and Slee (1977), who claimed that the acid α-glucosidase had two sites, one of which bound glycogen and maltose, and the other, 4-methylumbelliferyl-α-D-glucopyranoside. This finding would cast doubts on the usefulness of the 4-methylumbelliferyl conjugate as a substrate. The fluorogenic substrate, however, could be used for diagnosis, especially using liver and cultured cells (page 112) and was much more sensitive than maltose as was also found by Salafsky and Nadler (1973b).

(v) The β-glucosidase component deficient in Gaucher's disease.

Liver β-glucosidase had soluble and particulate components, which were separable by Sephadex G-150 gel filtration (page 153). The activity of the soluble component of spleen was much less than that of liver (page 154). In the two Gaucher livers studied, the β-glucosidase deficiency was detected in peak I (page 181) as predicted from studies using glucocerebroside as substrate (Ho, 1973). Apart from the component deficient in Gaucher's disease, there was another component in peak I (page 181), probably corresponding to the residual particulate enzyme reported by Owada et al. (1977) in certain Gaucher patients. The β-glucosidase activity of leucocytes and fibroblasts was eluted in the void volume of Sephadex G-150, no peak II being detected. Leucocytes had a considerable amount of the more neutral β-glucosidase activity, which was presumably the component associated with peak I and retained in Gaucher's disease, but there was little of this in fibroblasts. It is possible that the residual activity in Gaucher fibroblasts is an altered form of acid β-glucosidase as suggested by Turner et al. (1977). Interestingly, the pH profile of this component (page 176) was similar to that of the catalytic protein, factor C (Ho and O'Brien, 1971a).

(vi) Clinical heterogeneity of Gaucher's disease.

The markedly different clinical courses of the neuronopathic and non-neuronopathic forms of Gaucher's disease are not as yet satisfactorily explained. The liver of the case of infantile Gaucher's disease (R.L.) had non-specific β-glucosidase activity in both peaks I and II (page 180), so it was not possible to relate the severity of the disease in this patient to the activity of soluble and particulate fractions as attempted by Kanfer et al. (1975). Of two cases of adult Gaucher's disease investigated (pages 176,177), one had fibroblast β-glucosidase activity just below the control range, but, in the other case, activity was in the same range as that of infantile and juvenile patients. It was reported that in one case of adult Gaucher's disease, factor C was deficient in spleen (Ho and O'Brien, 1971a), whereas the activity of factor P, the effector protein, was raised. However, the determination of the activities of factors P and C in several cases of Gaucher's disease showed no clear difference between adult and infantile cases (Ho et al., 1977), three adult patients having no factor C and raised factor P in common with one infantile patient, but with another infantile patient having no factor P.

Interestingly, the activity of glucocerebrosidase is not markedly reduced in I-cell fibroblasts (Wenger et al., 1976), a property setting it apart from many other acid hydrolases and so this enzyme may have a secondary function in general lysosomal organisation. Niemann-Pick disease also exists in neurological and non-neurological forms (Frederickson and Sloan, 1972), but the enzyme associated with this disorder, sphingomyelinase, does have

low activity in I-cell fibroblasts (Wenger et al., 1976). It has been found (Peters et al., 1977), however, that sphingomyelin inhibits β-glucosidase activity and this may account for the storage of glucosyl ceramide in Niemann-Pick disease (Harzer et al., 1977) and might explain certain similarities between Gaucher's disease and Niemann-Pick disease. The difference between neuronopathic and non-neuronopathic Gaucher's disease is, however, more likely to be explained by a secondary effect than by some slight difference in the amount of residual activity.

(vii)Multiple specificity of β-glucosidase components.

When investigating the enzyme deficiency in Gaucher liver, Ockerman (1968) made use of the multiple specificity of soluble β-glucosidase. As well as having β-glucosidase activity, this component also had β-galactosidase and β-xylosidase activities. It was also reported that α-arabinosidase was deficient in Gaucher fibroblasts (Hultberg et al., 1973), as was β-fucosidase in Gaucher liver (Chester et al., 1976).

The results presented in this thesis are at variance with some of these claims. Using liver from cases of Gaucher's disease, $G_{M1}$ gangliosidosis and controls, it was found that all components of β-galactosidase, separated by Sephadex G-150 gel filtration had α-arabinosidase and β-fucosidase activity (page 166). All except the final β-galactosidase peak was deficient in $G_{M1}$ gangliosidosis liver (page 189); β-glucosidase and β-xylosidase activities were normal. The pH profile of β-galactosidase from control cultured skin fibroblasts (page 168), however, differed from those of α-arabinosidase and β-fucosidase, which were identical. A deficiency of α-arabinosidase and β-fucosidase in $G_{M1}$ gangliosidosis had previously been reported by van Hoof and Hers (1968) and Hindman and Cotlier (1972).

Liver β-glucosidase activity was eluted from Sephadex G-150 in two peaks, one of which was just before the first peak of β-galactosidase and the other, coincident with the final peak of β-galactosidase (page 166). The elution pattern of β-xylosidase coincided with that of β-glucosidase (pages 166, 188, 189), consistent with the structural similarity of the two substrates

(see the appendix, page 254). In Gaucher fibroblasts, the deficiency of β-xylosidase was more pronounced than that of β-glucosidase, but the activities of β-galactosidase, β-fucosidase and α-arabinosidase were normal (page 192). The multiple specificity of soluble β-glucosidase provides little information as to its natural function. It has been suggested (Chester et al., 1976) that this might be an "obsolete evolutionary remnant" with no present day function, consistent with its absence from some control livers (Butterworth et al., 1972; Cheetham et al., 1978). There was no evidence for the suggested enzymatic relationship (Chester et al., 1976) between Gaucher's disease and $G_{M1}$ gangliosidosis.

(viii)Methods for the removal of interfering components.

Methods were developed for the assay of specific enzyme components in tissues where other components were also found. At the outset of this work, Hex-A was assayed by heat (O'Brien et al., 1970) or pH (Saifer and Perle, 1974) inactivation, DEAE-cellulose chromatography (Young et al., 1970) or electrophoresis (Robinson and Stirling, 1968). The most useful quantitative method was found to be the DEAE-cellulose batch method (Dance et al., 1970), which gave results (page 27) closely in agreement with those by the DEAE-cellulose column method. The heat method underestimated the activity of Hex-A (page 27), probably due to some conversion of Hex-A to Hex-B (Tallman et al., 1974), but still reliably detected Tay-Sachs disease (page 70).

Techniques developed for the removal of non-specific α-glucosidases and β-glucosidases were by inactivation. Neutral and intermediate α-glucosidases were found to be susceptible to precipitation/inactivation in acetate buffer at pH 5.0 (page 95-97). The neutral α-glucosidase, residual in Pompe fibroblasts, had negligible activity at pH 4.0 (page 112) and treatment was unnecessary, whereas the intermediate component prevented reliable diagnosis of Pompe's disease in kidney and dextran isolated leucocytes (page 113). Using inhibitors, it was possible to estimate the relative activities of the acid and intermediate components in kidney (page 129) and leucocytes (page 132). Non-specific β-glucosidases of liver, not related to glucocerebrosidase activity, could be inactivated by preincubation with 50mM-sodium chloride buffered at pH 4.0 (page 186) enabling the reliable

diagnosis of Gaucher's disease in this tissue using 4-methylumbelliferyl-β-D-glucopyranoside. Recently it has been claimed (Turner et al., 1977) that pH 4.0 inactivation alone is sufficient to remove the non-specific β-glucosidase of leucocytes. This method was not fully reliable, but if the extract was preincubated at pH 3.0 a result similar to that obtained with 50mM-sodium chloride buffered at pH 4.0 was acheived (Butterworth and Broadhead, 1978). Peters et al. (1976) approached the problem of interfering non-specific β-glucosidases by assaying at more neutral pH and in the presence of sodium taurocholate, optimal conditions for the assay using natural substrate.

It would seem, therefore, that if account is taken of interfering non-specific hydrolases, artificial substrates may safely be used for the diagnosis of lysosomal storage disorders. In some instances, however, clinically normal individuals, usually related to affected patients have been found (Vidgoff et al., 1973; Dreyfus et al., 1975; Kelly et al., 1976; Wenger and Riccardi, 1976) to be deficient in a particular hydrolase activity. It would seem that in these individuals, the enzyme is altered so that it cannot effectively degrade the artificial substrate in vitro (Vidgoff et al., 1973; Dreyfus et al., 1975) or the natural substrate in vitro (Lott et al., 1976; Wenger and Riccardi, 1976), but can degrade the natural substrate sufficiently in vivo.

(ix) Suggestions for further research.

The relationship of tissue Hex-A to tissue Hex-B is now fairly well understood. However, the nature of the relationship of serum components to tissue components is still not clear and it is possible that investigation of Hex-S' will elucidate the situation. Similar studies to those of Beutler and Kuhl (1975) on the subunits of tissue hexosaminidases are required for serum hexosaminidases as are more detailed studies on the carbohydrate content of serum and tissue forms.

It has been possible to estimate acid α-glucosidase in most tissues using a variety of techniques. Some knowledge of other α-glucosidase components has been obtained. One of these was present in amniotic fluid but not in other enzyme sources. The relationship of this component to other α-glucosidases (e.g. urine α-glucosidase (Salafsky and Nadler, 1973a)) should be studied.

Further investigation of the relationship of the residual β-glucosidase of Gaucher fibroblasts and liver peak I to glucocerebrosidase activity is required. It is unlikely that the clinical heterogeneity of Gaucher's disease is due to slight differences in the amount of residual β-glucosidase activity and a different approach may be necessary. One possibility is that the β-glucosidase detected either by artificial substrate or natural substrate shares a common subunit with an enzyme of different substrate specificity.

For example

| β-glucosidase | enzyme X |
|---|---|
| $\alpha_n\beta_n$ | $\gamma_n\beta_n$ or $\beta_{2n}$ |

A deficiency of this subunit (β) would lead to an absence of β-glucosidase and enzyme X resulting in neuronopathic Gaucher's disease, whereas a patient deficient in α subunits would only lack β-glucosidase giving rise to non-neuronopathic Gaucher's disease. A similar model could explain the clinical differences between adult and infantile Pompe's disease. If the model were correct for either of these diseases, the amount of effort devoted to understanding the hexosaminidase system will have been justified.

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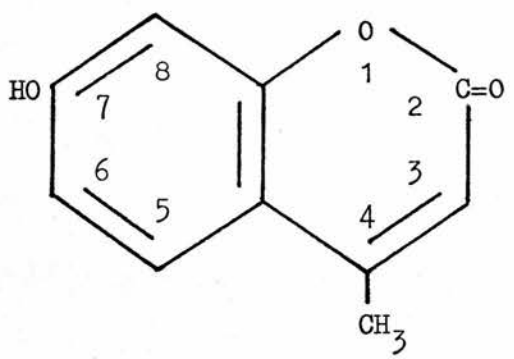
# APPENDIX

Fluorimetric Enzyme Assays.

Acid hydrolases were assayed by measuring the amount of the aglycone, 4-methylumbelliferone released from a particular 4-methylumbelliferyl conjugate. The absorbance and fluorescence characteristics of the 4-methylumbelliferyl conjugate and the 4-methylumbelliferone anion are quite different (Leaback, 1974), the former having peak absorbance at 330nm and peak fluorescence at 380nm, and the latter at 365nm and 445nm respectively.

In the experiments described in this thesis, cell or tissue extract was mixed with buffered substrate and after a measured time interval the assay was stopped with 0.1M-sodium carbonate. This not only inactivated the enzyme, but also ionised the liberated 4-methylumbelliferone to its anion. The fluorescence was measured using either a Perkin-Elmer 203 or Perkin-Elmer 1000 spectrofluorimeter, which had been standardised using quinine bisulphate (1µg/ml) in 0.05M-$H_2SO_4$.




4-Methylumbelliferone

Conjugates are linked at the 7 position.

Protein Determination.

Protein was determined by a modification of the method of Lowry et al. (1951) as follows:-

Reagents.

A 10% $^{w}/_{v}$ $Na_2CO_3$ in 0.5M-NaOH.

B 0.5% $^{w}/_{v}$ $CuSO_4.5H_2O$ in 1% sodium tartrate.

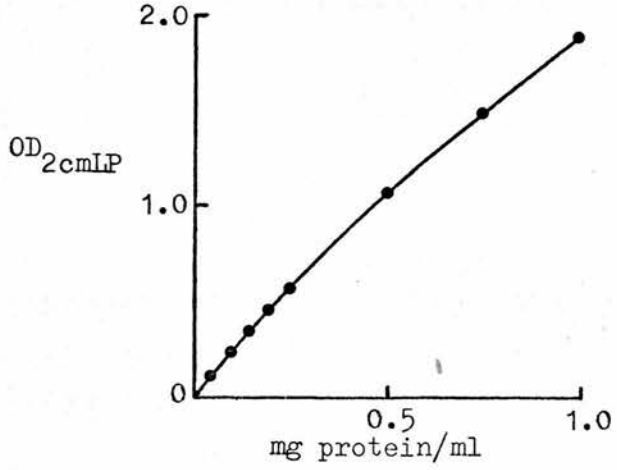
C 9ml A + 1ml B, prepared just before use.

D Folin-Ciocalteu reagent (Sigma Chemical Co.) diluted 1:5 with distilled water before use.

Protein standard: lyophylised bovine serum albumin (Sigma Chemical Co.), 0.25mg/ml in distilled water.

Procedure.

Solution C (0.3ml) was added to 0.3ml of sample (0.05ml extract + 0.25ml distilled water) and mixed on a vortex mixer. After 10 minutes, 0.3ml of solution D was added with thorough mixing on a vortex mixer and left at room temperature for 30 minutes. The extinction at 650nm was read against a no protein blank.




Standard curve for determination of protein as above.

Tissue Culture.

All tissue culture in this thesis was carried out by the tissue culture and cytogenetics staff of the Pathology Department of the Royal Hospital for Sick Children, Edinburgh. Techniques used are outlined below.

Requirements.

Tissue culture medium.

The tissue culture medium (nominal volume of 5 litres) was made up as follows:-

| | |
|---|---|
| Ham's F10 concentrate (10x) (Flow Labs. Ltd.) | 400ml |
| Distilled deionised water | 3460ml |
| L-glutamine (200mM) (BDH) | 40ml |
| Fetal bovine serum (Flow Labs. Ltd.) | 720ml |
| Kanamycin sulphate BPC ("Kanasyn", Winthrop Labs.) | ½ bottle |
| or Penicillin-streptomycin ("Crystamycia", Glaxo Labs.) | 1 bottle |
| $NaHCO_3$ (4.4%) "Analar" | 100ml |

The medium was prepared non-sterile and filtered through a "Millipore" filter with a pore size of 0.22µ, then sterility tested. For amniotic fluid cell primary cultures, the medium was supplemented with more fetal bovine serum, thus raising the serum concentration from 15% to 30%.

Culture vessels.

Cells were cultured in baby's feeding bottles of 225ml capacity and 40$cm^2$ growth surface area. Amniotic fluid cell primary cultures were in Nunclon-delta Petri dishes of 50mm diameter.

Cell culture.

Skin biopsies.

The skin was cut up into small pieces in thrombin. Approximately eight drops of chicken plasma were dropped onto one face of a baby's feed bottle and a small piece of skin was placed on each. Excess liquid was removed using a pasteur pipette. When these drops had clotted, medium (10ml) was added and the bottle was gassed with 5% $CO_2$ in air. Outgrowths of fibroblasts spread from the pieces of skin and these were subcultured.

Amniotic fluid and urine cells.

Cells were precipitated by centrifugation at 300g for 10 minutes. The supernatant was poured off and the cells were resuspended in medium, supplemented with 30% fetal bovine serum. The cells, suspended in medium, were put into Petri dishes (approx. 5ml in each). The Petri dishes were placed in anaerobic jars (modified McIntosh and Fildes design) and gassed with 5% $CO_2$ in air.

Subculture.

Cells were removed from the culture vessel using 0.25% $^{w}/_{v}$ trypsin solution (Trypsin 1:250, Difco Labs.) in phosphate buffered saline (Dulbecco "A", Oxoid Ltd.). The cells were centrifuged, resuspended in medium and transferred to two culture vessels.

Cells were incubated at 37°C at all times.

Preparation of Cultured Cells.

Requirements.

Monolayer of cultured cells.

Trypsin-versene solution, constituted as follows:-

| | |
|---|---|
| Trypsin 1:250 (Difco labs.) | 2.5% W/v |
| EDTA (BDH) | 0.4% W/v |

dissolved in phosphate buffered saline (PBS).

Phosphate buffered saline made from Dulbecco "A" tablets (Oxoid Ltd.).

Procedure.

The medium was poured off the cells, which were then rinsed once with PBS. Into each culture vessel was pipetted 3.6ml PBS and 0.4ml trypsin/versene solution and after gentle mixing the vessel was left on the bench with the cells on the bottom surface. After a period of time, which depended on the cell type and the nature of the surface of the culture vessel (plastic or glass), the cells lifted and were transferred to a 10ml conical centrifuge tube. The cells were sedimented by centrifugation (400g for 5 minutes) and washed twice with PBS.

Dextran Isolated Leucocytes.

Dextran (1.4ml) ("Dextraven 110", Fisons Ltd.) was put into a 10ml lithium heparin tube. Venous blood (8.5ml) was run into the tube and mixed gently by inverting several times. Any bubbles which had formed on top were burst using a red hot spatula. The tube was left for one hour at $4^{o}C$.

After one hour, the top layer was removed leaving the erythrocytes at the bottom of the tube. The top layer contained polymorphonuclear cells, the lymphocytes having been sedimented on top of the erythrocytes. After centrifugation (900g for 5 minutes) of the top layer, a pellet of cells still heavily contaminated with erythrocytes was obtained. The pellet was mixed vigorously with 0.5ml of sodium chloride solution (0.2% $^{w}/v$) for thirty seconds, after which isotonicity was restored by the addition of 0.5ml of 1.62% $^{w}/v$ sodium chloride. After centrifugation (900g for 5 minutes), this lysis step was repeated and following a third centrifugation, a pellet of leucocytes relatively free of contamination from erythrocytes was obtained.

Preparation of Lymphocytes (Boyum, 1968).

Requirements.

Siliconised glass 10ml centrifuge tubes.

The tubes were rinsed with dimethyldichlorosilane solution, 2% in 1,1,1-trichloroethane, (BDH), drained then dried in an oven. The tubes were then washed out to remove any excess hydrochloric acid, rinsed in distilled water and dried in an oven.

Balanced salt solution (BSS).

Two solutions (A and B) were made up as follows:-

Solution A

| | |
|---|---|
| D-glucose (anhydrous) | 0.1% $^{w}/_{v}$ |
| $CaCl_2 . 2H_2O$ | $5.0 \times 10^{-5}$M |
| $MgCl_2$ | $9.8 \times 10^{-4}$M |
| KCl | $5.4 \times 10^{-3}$M |
| TRIS | 0.145M |

When one litre was required, the volume was initially made up to 950ml and 1M-HCl was added until pH 7.6 was reached, after which the solution was made up to a litre.

Solution B

| | |
|---|---|
| NaCl | 0.14M |

Balanced salt solution was a mixture of one part of solution A with nine parts of solution B.

Ficoll-Paque (Pharmacia Fine Chemicals).

An aqueous solution of Ficoll 400 (5.7g) and diatrizoate sodium (9g) per 100ml.

Heparinised blood.

Procedure.

To each centrifuge tube was added 3ml of Ficoll-Paque. The heparinised blood was diluted 50:50 with BSS and after thorough mixing, 4ml was layered gently onto the Ficoll-Paque using a Pasteur pipette. This was then centrifuged at 400g for 40 minutes at ambient temperature.

After centrifugation, the lymphocytes were at the interface between the blood-BSS mixture and the Ficoll-Paque. The erythrocytes and polymorphonuclear cells had been precipitated to the bottom of the tube. The upper layer, which now contained diluted plasma and platelets, was removed. The lymphocytes were transferred to another centrifuge tube, washed twice with BSS and centrifuged at 900g for 5 minutes. The lymphocytes were retained as a cell pellet.

Preparation of DEAE-cellulose.

DE-52 (approx. 25g) was washed into a 250ml centrifuge bottle with 0.1M-HCl (100ml). A magnetic 'flea' was added to aid mixing and the slurry was degassed for 5 minutes under reduced pressure. The pH was adjusted to pH 6-7 with M-$Na_2HPO_4$ and the resin was sedimented at 250 r.p.m. for 5 minutes. The supernatant was decanted off and the resin was twice resuspended and washed in 50mM-sodium phosphate buffer, pH 6.0 (or 6.8). The resin was finally suspended in 10mM-sodium phosphate buffer, pH 6.0 (or 6.8), containing sodium azide (0.02% $^{w}/v$) and Triton X-100 (0.05% $^{v}/v$). For the DEAE-cellulose batch method, the slurry was adjusted to 50% $^{v}/v$ in this buffer.

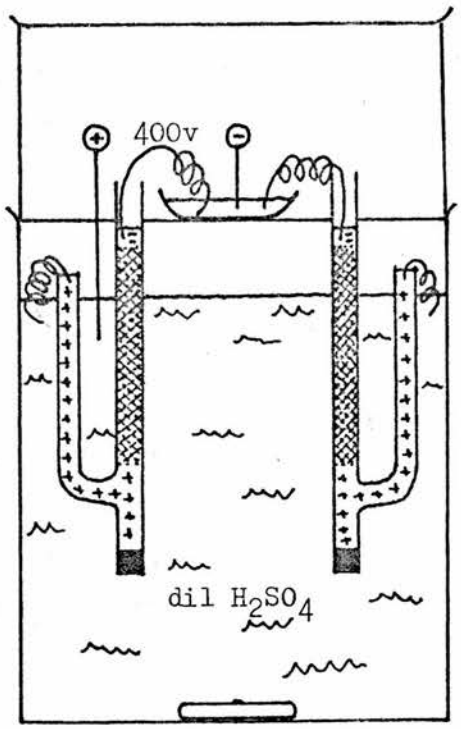
Isoelectric Focusing in a Sucrose Gradient.

The method was a modification of that of Godson (1970). The "J-tubes" were as specified by Godson. Solutions were made up as follows:-

| | |
|---|---|
| Anode solution | 12g sucrose, 14ml distilled water, 0.2ml orthophosphoric acid |
| Cathode solution | 1ml ethanolamine, 50ml distilled water |
| Heavy gradient | 2.8g sucrose, 4.2ml distilled water, 0.06ml 10% $^{v}/_{v}$ Triton X-100, 0.75ml 10% ampholine |
| Light gradient | sample made up to 6ml with distilled water, 0.06ml 10% $^{v}/_{v}$ Triton X-100, 0.25ml 10% ampholine |
| Electrolyte | dil. $H_2SO_4$ in tap water |

The anode (14ml) was run into the short arm of the J-tube. The sucrose gradient was made using a gradient mixer constructed from two ten ml syringe barrels. The heavy gradient (4.5ml) was first added to the mixing chamber and allowed to fill the exit tube and the tube joining the two chambers before being clamped. The light gradient (5ml) was then run into the second chamber. The gradient, heavy to light, was then run gently down the long arm of the J-tube. Cathode (approx. 2ml) was then layered on top of the gradient.

The anode and cathode were connected to the power pack by platinum wires dipped into the electrolyte, which was in a small dish at the cathode and was used as a coolant for the J-tubes at the anode (see diagram, next page). The electrolyte was connected by platinum wires to the power pack. The voltage was raised




Schematic diagram of J-tube apparatus for sucrose gradient isoelectric focusing.

step-wise to prevent excess current until 400v was reached. This voltage was maintained at 4°C for 18 hours.

On completion, an airtight connection was attached to the short arm and heavy sucrose (anode solution) was gently pumped in, thus displacing the sucrose gradient from the long arm. Fractions of between 8 and 12 drops were collected and the pH of each fraction was read at 4°C.

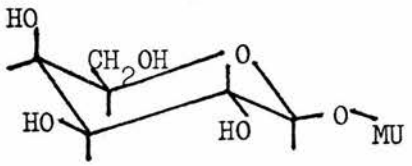
Glucose Oxidase Method.

Reagents were added to an LP3 tube (Luckham Ltd.) as follows:-

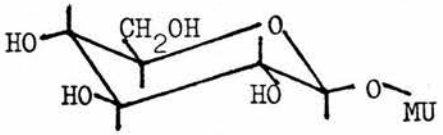
| | |
|---|---|
| Sodium phosphate buffer (0.1M), pH 7.0 containing o-dianisidine-diHCl (0.1mg/ml) and saturated with oxygen. | 0.60ml |
| Sample or distilled water for blank. | 0.30ml |
| Horseradish peroxidase Type II (Sigma Chemical Co.) (2mg/ml). | 0.01ml |
| Glucose oxidase Type V from *Aspergillus niger* (Sigma Chemical Co.) | 0.01ml |

After 45 minutes at ambient temperature, concentrated hydrochloric acid (0.45ml) was added and the extinction at 530nm read against a no glucose blank. With each batch a standard curve for glucose was obtained.

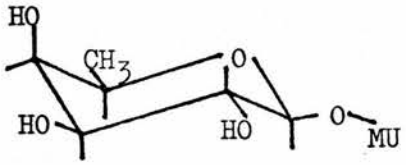
Structural Comparison of Substrates Attacked by the Same Enzyme.



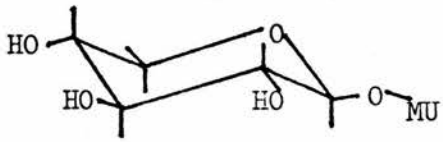

4-MU-β-D-galactopyranoside



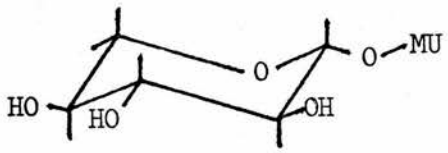

4-MU-β-D-glucopyranoside




4-MU-β-D-fucopyranoside

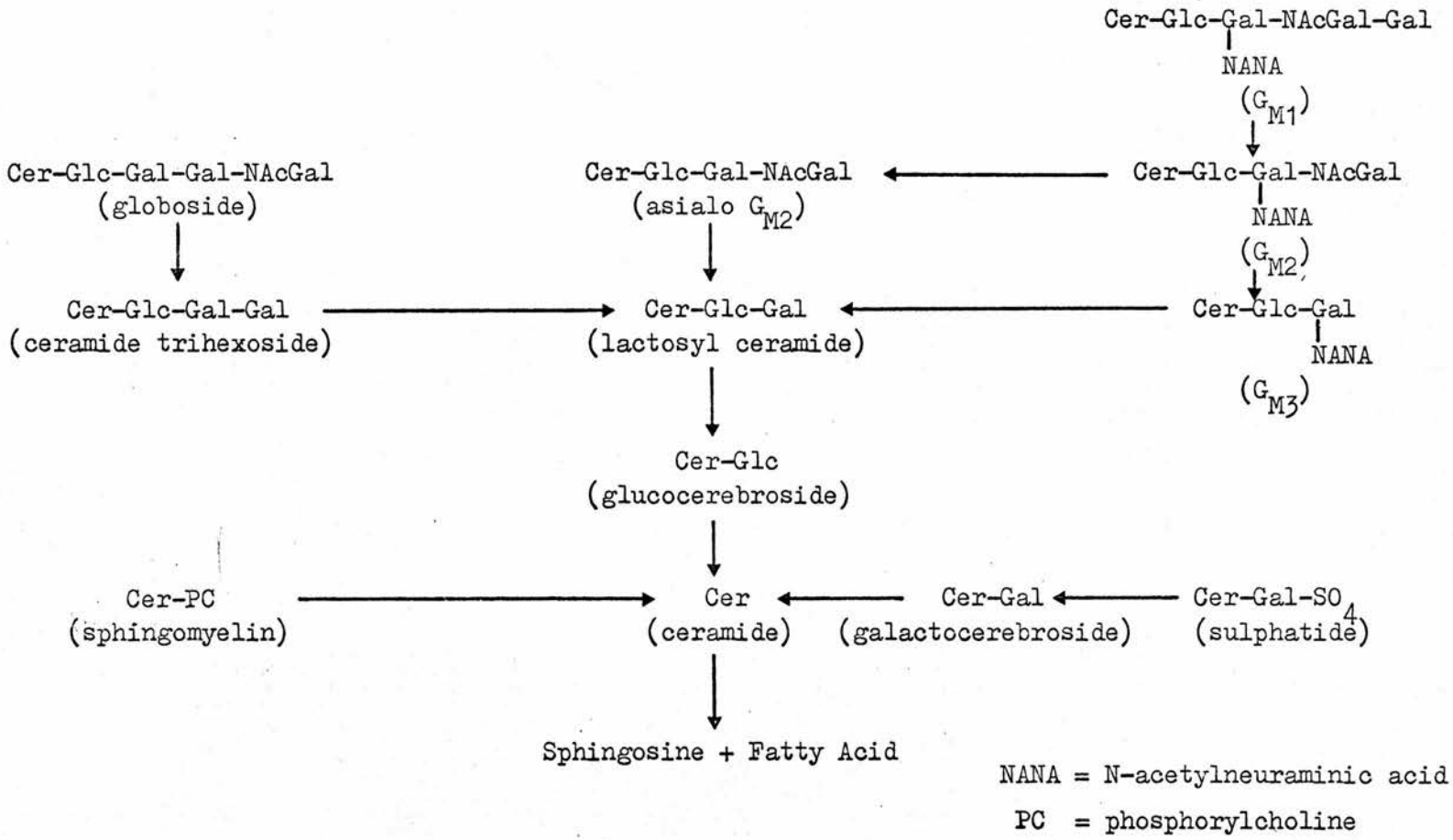



4-MU-β-D-xylopyranoside




4-MU-α-L-arabinopyranoside

Degradative Pathways of Sphingolipids.



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